Bone repair potential of human amniotic fluid stem cells

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Declaration of authorship

I, Anna Maria Ranzoni, declare that this thesis titled “Bone repair potential of human amniotic fluid stem cells” and the work presented in it are my own.

I confirm that:

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- No part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution.
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Abstract

The amniotic fluid is a rich source of fetal mesenchymal stem cells with broad differentiation capacity. Human amniotic fluid stem cells (AFSCs) have a high expansion potential, fast growth kinetics and harbour therapeutic potential to treat a variety of conditions. Moreover, they are easily isolated at mid-trimester or at delivery and can be used without ethical restrictions. Osteogenesis imperfecta (OI) is a genetic disease characterised by bone fragility, due to production of abnormal collagen type I.

In this study, human AFSCs were transplanted into immunocompetent *oim* mice (OI mouse model, n=28) at birth. Bones were harvested after 8 weeks and analysed for mechanical properties, micro-structure, engraftment of donor cells and gene expression. Non-transplanted *oim* and wild-type mice were used as controls.

Human AFSC injection decreased bone fracture rate and increased bone strength. Donor cells migrated to the bones, engrafted into sites of active bone formation and appeared to differentiate into osteoblasts, producing normal collagen. Moreover, transplantation improved the microarchitecture of the bones, although bone volume remained unaffected. Transplantation also promoted endogenous osteogenesis, with mouse genes involved in osteoblast differentiation and skeletal development significantly up-regulated, compared to non-transplanted mice.

As a side project, a protocol for the isolation and differentiation of human fetal osteoblasts from the calvaria was optimised and a biobank of 24 samples, from gestational ages ranging from 9 to 21 weeks post-conception, was established.

The data presented in this thesis indicate that human AFSCs are a promising source for cell therapy in OI. Donor cells may exert their therapeutic effects both by normalising the ECM and by influencing the maturation of resident osteoblasts. Ongoing work is focused on the further understanding of the mechanism of action of donor cells, using co-culture experiments with human AFSCs and human fetal osteoblasts.

Statement of Impact

Data presented in this thesis provide insights in support of cell therapy approaches using fetal mesenchymal stem cells (MSCs) for the treatment of osteogenesis imperfecta (OI).

Research from this and other labs showed that the transplantation of human fetal MSCs has therapeutic effects in mouse models of the disease. These studies, in addition to few transplantations carried out in children, paved the way for the first clinical trial for prenatal transplantation of stem cells for OI.

Building from these previous studies, the experiments described here for the first time suggest that human amniotic fluid stem cells have therapeutic effects on a mouse model of brittle bone disease and therefore represent a promising source of stem cells for the treatment of the disease.

Moreover, results presented in this thesis open new questions regarding the mechanism of action of human amniotic stem cells, which is of interest for future research.

Finally, the protocol for the isolation of human fetal osteoblasts presented in this thesis can benefit the research community as it allows easy isolation and robust differentiation of human osteoblasts that can be used as an *in vitro* model of human bone.

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Chapter 1

Introduction

1.1 Bone composition and function

Bone is a mineralised connective tissue with structural, metabolic and synthetic functions. The main role of bones is to provide structural support to the body, allowing movement and protecting internal organs. Bone also acts as a reservoir for minerals, essential for the chemical homeostasis of the body. Its extracellular matrix (ECM) contains more than 90% of the total phosphate and calcium ions in the human body, in addition to numerous growth factors including transforming growth factor β (TGF-β), insulin-like growth factors (IGFs) and bone morphogenetic proteins (BMPs) (Baylink et al., 1993; Flynn, 2003). Finally, bone also exerts a synthetic function, with the bone marrow being the site of haematopoiesis, the process in which hematopoietic cells are formed and differentiate.

1.1.1 Bone development

During mammalian fetal development, bones are formed in two distinct ways. Most bones in the human skeleton, including vertebrae and long bones of the limbs, develop by endochondral ossification, a process which requires a cartilage intermediate. The flat bones of the skull, the mandible and the sternum, on the other hand, generate through intramembranous ossification, without the involvement of a cartilage intermediate.

Endochondral ossification starts with the condensation and differentiation of mesenchymal stem cells (MSCs) into chondrocytes, through the local expression of the transcription factor SOX9 (SRY-box 9). Chondrocytes secrete cartilage-specific matrix and produce a cartilaginous model of the forming bone. Subsequently, chondrocytes become hypertrophic and undergo apoptosis, while the deposited cartilage starts being mineralised. The cartilaginous model is then invaded by blood vessels due to the local release of growth factors, including VEGF (vascular endothelial growth factor), while expression of RUNX2 (runt-related transcription factor 2) drives the differentiation of MSCs into osteoblasts. The cartilage model acts as a template for osteoblasts to replace chondrocytes and deposit collagen type I (Bruder and Caplan, 1989). The newly formed trabecular bone gradually replaces

completely the cartilage framework. From this primary centre of ossification, bone grows towards the ends of the structure, where secondary ossification centres develop. A small portion of cartilage remains where the primary and secondary centres meet and forms the growth plate of long bones, allowing the bone to grow further, until development ends and the growth plate is replaced by mineralised tissue (Mackie et al., 2008; Gilbert, 2014).

“Intramembranous” ossification begins with the proliferation and condensation of MSCs into compact structures of connective tissue, where blood vessels develop as well. Upon expression of RUNX2, the MSCs found within the structures differentiate into osteoblasts and start secreting collagen and depositing osteoid. The collagenous matrix gradually mineralises and the osteoblasts that remain trapped inside it become osteocytes. As development continues, mineralised spicules form from the centre of ossification, while a thin layer of condensed MSCs surrounds the newly formed bone, forming the periosteum (Gilbert, 2014).

1.1.2 Stromal fraction of bones

Bones contain a rich connective compartment, comprising of many stromal cell types which support haematopoiesis and contribute to bone homeostasis. The bone marrow is a highly vascularised tissue consisting of islands of haematopoietic cells mixed with adipose cells (Anthony and Link, 2014). Marrow adipose tissue can make up to 70% of the total bone marrow volume and is thought to be one of the main energy storages of the body (Suchacki et al., 2016). In addition to adipocytes, the stromal compartment of the bone marrow also includes mesenchymal stem cells, pre-osteoblasts, osteoblasts and chondrocytes. The characteristics of osteoblasts, osteocytes and osteoclasts, the three main cell types found in bone, each exerting a specific function to contribute to bone homeostasis, are discussed below.

1.1.3 Osteoblasts and bone formation

Osteoblasts, or bone forming cells, are characterised by cuboidal/cobblestone morphology and have been estimated to represent the 4-6% of the total cells present

in human bones (Florencio-Silva et al., 2015). Osteoblasts have the function of depositing collagen and forming the osteoid. When osteoblasts are not actively secreting proteins, they are found in a quiescent state, in which they become flat-shaped bone-lining cells and cover the bone surface (Miller et al., 1989). Osteoblasts derive from MSCs, through the strictly regulated and timed expression of osteogenic genes. The master regulator of osteogenic commitment is RUNX2, as demonstrated by the lack of differentiated osteoblasts in RUNX2-deficient transgenic mice (Komori et al., 1997). RUNX2 expression leads to the upregulation of osteoblast-specific genes including collagen type I (COL1A1 and COL1A2) and alkaline phosphatase (ALP), leading to the differentiation of early osteoblasts. Subsequently, the expression of genes encoding proteins of the extracellular matrix (ECM), such as osteocalcin (BGLAP) and bone sialoproteins, indicates the terminal differentiation of osteoblasts (Komori et al., 1997).

Two steps are required for the formation of bone ECM by osteoblasts. The first stage concerns the secretion by osteoblasts of the organic component of the matrix, composed of collagen type I, non-collagenous proteins, including osteopontin, osteonectin, osteocalcin and bone sialoprotein, and proteoglycans, including decorin and biglycan. The second step is the mineralisation of the organic matrix. Initially, osteoblasts release vesicles containing calcium ions, which are deposited on the matrix by binding to proteoglycans (Anderson, 2003). Osteoblasts also produce alkaline phosphatase, which degrades phosphate-containing compounds releasing phosphate ions. Subsequently, calcium and phosphate ions nucleate inside the vesicles forming hydroxyapatite, which is then released to form the mineral component of bone matrix (Glimcher, 1987). Mineralisation depends on the availability of calcium and phosphate ions and is negatively regulated by pyrophosphate (Hajjawi et al., 2014; Orriss et al., 2016).

1.1.4 Osteocytes and mechanosensing

Following bone formation, a subpopulation of osteoblasts further differentiates into osteocytes, which are estimated to represent about 90% of the total number of bone cells. Located in bone lacunae and embedded into the matrix, osteocytes have a

remarkably long life-span and can live up to years (Franz-Odendaal et al., 2006). Osteocytes are characterised by numerous cytoplasmic processes elongating in the lacunar canaliculi, that confer the peculiar dendritic morphology (Dallas et al., 2013). The cytoplasmic elongations allow osteocytes to interact with other osteocytes by gap junctions and with osteoblasts, contributing to cell signalling and to the exchange of oxygen and nutrients coming from the vasculature. It is thought that, through this extensive network, osteocytes exert the function of sensing mechanical loading of the bone, thus contributing to the bone adaptation to such stimuli, although the exact mechanism has not been fully understood (Klein-Nulend et al., 1995; Rochefort et al., 2010; Al-Jazzar et al., 2016). Osteocytes regulate bone mineralisation, through the expression of sclerostin, which inhibits osteoblast activity, and the production of RANK ligand (RANKL), which promotes osteoclast formation (Bonewald, 2011; Xiong et al., 2015). Moreover, osteocytes are a source of pyrophosphate, which inhibits mineralisation, and secrete signalling molecules such as nitric oxide and prostaglandins (Dallas et al., 2013; Orriss et al., 2016).

1.1.5 Osteoclasts and bone resorption

Osteoclasts, or bone resorbing cells, are large multinucleated cells deriving from mononuclear cells of the hematopoietic lineage. Osteoclast formation starts with the secretion by osteoblast progenitors and osteoblasts of M-CSF (macrophage colony-forming factor) and RANKL (RANK ligand). M-CSF promotes cell survival and proliferation while RANKL, interacting with its receptor RANK located on the surface of osteoclast precursors, stimulates osteoclast differentiation (Teitelbaum, 2000). On the other hand, osteoblasts produce osteoprotegerin (OPG), which prevents RANKL from binding to its receptor RANK, thus inhibiting osteoclast differentiation (Hofbauer et al., 2000). The RANKL/RANK/OPG pathway therefore plays a key role in the regulation of osteoclastogenesis. The main role of osteoclasts is the degradation of bone matrix, a process known as bone resorption. In a physiological context, bone resorption is coupled with bone formation in achieving the constant renewal of the tissue (**Figure 1**).

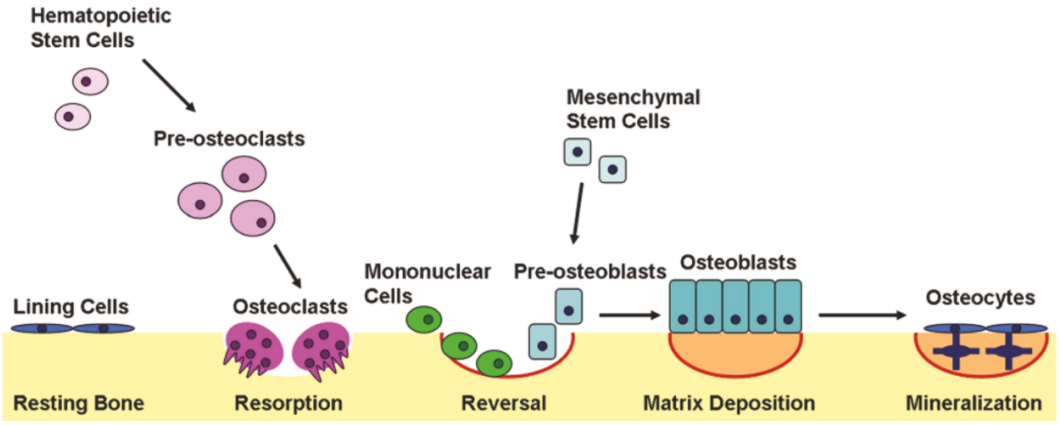


Figure 1. Bone remodelling.

Bone remodelling is a complex process involving two main steps: bone resorption by osteoclasts and bone formation by osteoblasts. (Kapinas and Delany, 2011).

Bone remodelling is the life-long dynamic process resulting from the anabolic and catabolic activities of osteoblasts and osteoclasts. Bone resorption is a complex multistep process, which starts with osteoclast polarisation. The part of the osteoclast in contact with the bone matrix becomes characterised by a ruffled border, composed by numerous microvilli, and a sealing zone, to tightly attach to the bone matrix to be degraded. Bone resorption occurs via the release of protons across the ruffled border, through a H^+-ATPase, which causes a local acidification and the consequent degradation of hydroxyapatite crystals (Arana-Chavez and Bradaschia-Correa, 2009). Lysosomal enzymes produced by osteoclasts, such as cathepsin-K, are responsible for the degradation of the organic component (Troen, 2004). More recently, osteoclasts have been described as cytokine-secreting cells and the name clastokines was coined to refer to the numerous factors produced by osteoclasts, most of which are involved in the regulation of osteoblast activity and in the modulation of bone remodelling (Drissi and Sanjay, 2016).

1.1.6 Collagen type I structure and biosynthesis

Collagen type I accounts for 90% of total proteins found in bones, to which it provides mechanical strength. Collagen type I is a 300nm long heterotrimer formed by two α1(I) and one α2(I) chains, encoded by *COL1A1* and *COL1A2* genes respectively, that are defined by the repetition of the Gly-X-Y tri-peptide repeat. Glycine is always found

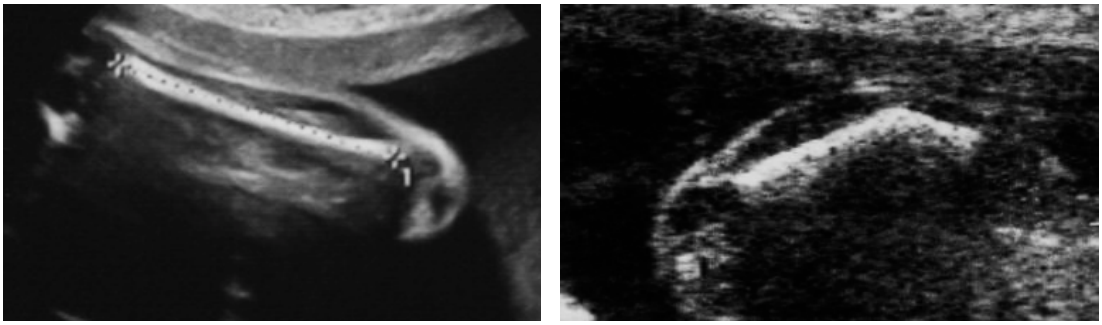
in the first position of the triplet for steric reasons; its side chain is the only one small enough to fit into the coiled coil structure of the collagen triple helix. X and Y can be any amino acids, although hydroxyl-proline is often found in the third position to confer stability to the trimeric molecule, by allowing the twisting of the molecule (Rich and Crick, 1961; Brodsky and Persikov, 2005).

Collagen biosynthesis is a complex process which takes place in different compartments of the cell and involves many proteins. COL1A1 and COL1A2 transcripts are first translated into the pro-collagen proα1(I) and proα2(I) chains, in the endoplasmic reticulum. Pro-collagen is then assembled into a left-handed triple helix of 1,014 amino acids, starting from the C-terminus, which is characterised by the presence of disulphide bonds. Pro-collagen assembly is assisted by a number of chaperone proteins found in the endoplasmic reticulum, including serpin H1 and the multi-protein prolyl 3-hydroxylation complex (Marini et al., 2007).

Before assembly is complete, pro-collagen chains are also subject to several post-translational modifications. The prolyl 3-hydroxylation complex is responsible for the 3-hydroxylation of proline residues, specifically α1(I)Pro986 and α2(I)Pro707, while P4H (L-proline trans-4-hydroxylase) and PLOD (procollagen lysyl-hydroxylase) carry out the 4-hydroxylation of proline residues and the hydroxylation of lysine residues, respectively (Myllyharju and Kivirikko, 2004). Proline hydroxylation, a reaction that requires ascorbic acid and iron ions as a co-factors and oxygen, is particularly important because it has been shown to stabilise the collagen molecule. (Hutton et al., 1967). Upon secretion, proteinases cleave the C- and N-terminal globular domains of pro-collagen, forming tropo-collagen. The removal of the C-terminus acts as a signal for the final assembly of mature collagen molecules into 50 µm fibrils. The organisation of individual fibrils into collagen fibres is achieved by covalent cross-linking between adjacent lysine and hydroxylysine residues, catalysed by lysyl oxidases (Kadler et al., 1987).

1.2 Osteogenesis imperfecta

Osteogenesis Imperfecta (OI), also called brittle bone disease, comprises a heterogeneous group of congenital diseases that overall affect 1 in every 15-20,000 live births (Forlino and Marini, 2016). OI is manifested by thin and brittle bones that are more fragile and prone to frequent fractures, even as a result of mild trauma. Other hallmark features comprise growth deficiency, vertebral compression, bowing of long bones, joint hypermobility, dentinogenesis imperfecta and macrocephaly. Depending on disease severity, non-skeletal features may also be present, including degenerative hearing loss, reduced pulmonary function, blue-coloured sclerae and defects of cardiac valves (Kuurila et al., 2000; LoMauro et al., 2012). Histomorphometric analyses in OI show a generalised osteopenia with long bones characterised by reduced cortical and trabecular thickness (Rauch et al., 2000). OI has prenatal onset and its most severe forms can be detected during second and third trimester ultrasound scans, when fractures and bowing of long bones can be visualised (**Figure 2**).



Healthy femur OI femur

Figure 2. Prenatal diagnosis of OI.

Prenatal ultrasound scan comparing femurs from a healthy fetus and a fetus with severe OI. Courtesy of Prof. Anna David (UCL Institute for Women's Health).

1.2.1 Genetic origin

OI is a connective tissue disorder originating from abnormal production of collagen type I. OI was initially considered an autosomal dominant disorder caused only by mutations on genes encoding for the two types of α-chain forming collagen type I,

namely COL1A1 and COL1A2. In the last decade, however, mutations in other 14 genes have been identified as cause of recessive forms of the disease (Morello et al., 2006; Lim et al., 2017). Now more than 1,500 mutations have been linked to OI, with about 85-90% of cases caused by structural mutations in the two collagen genes and the remaining caused by mutations on genes involved in the biosynthesis of collagen type I, including its post-translation modifications and folding (Rauch and Glorieux, 2004; Forlino and Marini, 2016).

The most frequent mutations observed are glycine substitutions in the helical domains of collagen type I, which cause an impaired trimer assembly. Glycine substitution in the α1 chains lead to more severe phenotype and even to perinatal lethality, while those mutations on the α2 chain are associated with milder features (Lim et al., 2017). Mutations in genes encoding for the α-chains of type I collagen cause dominant forms of OI, with qualitative or quantitative defects. Heterozygous null mutations in COL1A1 lead to degradation of the abnormal transcript and, as a result, only half of the normal amount of collagen type I is produced. Mutations in COL1A2, on the other hand lead to a variety of phenotypes and severities, due to the accumulation in the extracellular matrix of collagen homotrimers that fail to correctly assemble (Forlino et al., 2011). Qualitative structural defects in the collagen chains have been linked with more severe phenotypes than quantitative defects in which less normal collagen is produced (Forlino and Marini, 2016).

Procollagen is subject to a number of post-translational modifications that are necessary for its correct folding, secretion and interaction with other ECM proteins. Mutations in genes involved in this processing generate a variety of OI phenotypes with recessive inheritance, that are commonly referred to as non-classical OI syndromes. Most mutations causing non-classical OI are found on genes involved in the formation of the prolyl 3-hydroxylation complex, a multi-protein complex responsible for the 3-hydroxylation of specific proline residues in different types of collagen, necessary for its folding (Marini et al., 2007). In particular, mutations in *CRTAP* and *LEPRE1* genes cause very severe OI phenotypes, characterised by osteochondrodystrophy, since the complex is also involved in cartilage formation (Barnes et al., 2006; Morello et al., 2006; Cabral et al., 2007). Other genes

participating in collagen crosslinking are known to harbour mutations leading to OI and related disorders. *PLOD2* and *FKBP10* encode for proteins involved in the conversion of lysine residues into hydroxylysine, necessary for the establishment of crosslinks (Gjaltema et al., 2016). Mutations on these genes results in a specific type of OI with joint contractures, known as Buck syndrome (Ha-Vinh et al., 2004). SerpinH1 is a chaperone protein which contributes to collagen translocation between the endoplasmic reticulum and the Golgi apparatus, through the stabilisation of the collagen triple-helix (Koide et al., 2006). Mutations in this gene cause severe recessive OI due to collagen aggregation and impaired secretion (Christiansen et al., 2010).

Osteoblast differentiation is crucial for collagen deposition and mineralisation and genes of the Wnt signalling pathway have been linked to OI. *WNT1* heterozygous mutations are associated with early-onset osteoporosis, while homozygous null mutations lead to OI (Pyott et al., 2013). Finally, *CREB3L1* encodes a endoplasmic reticulum-stress transducer which translocates into the nucleus and is responsible for the activation of the *COL1A1* promoter (Murakami et al., 2009).

1.2.2 Pathogenesis

As a consequence of abnormal collagen production, fibrils are unable to fold in the correct heterotrimeric conformation and to interact with non-collagenous proteins. Moreover, collagen chains that fail to assemble activate the unfolded protein response in the endoplasmic reticulum, leading to protein degradation (Boot-Handford and Briggs, 2010). This causes the formation of a disorganised ECM, unable to support the normal apposition of minerals. The resulting disordered mineralization contributes to bone fragility observed in OI (Sweeney et al., 2008).

At a cellular level, OI manifests itself by an increased number of osteoblasts that fail to reach full maturity and individually produce less bone (Rauch et al., 2000). Osteoblasts derived from OI patients produce lower levels of osteonectin and proteoglycans and higher levels of fibronectin and hyaluronan (Fedarko et al., 1996). In addition, the ECM of OI patients is characterised by aberrant levels of mineralisation, which contribute to bone brittleness and fragility (Roschger et al.,

2008; Rauch et al., 2010). Moreover, OI is characterised by high osteoclast activity and, overall, this results in an increased bone turnover with no bone mass gain (Forlino et al., 2011; Biggin and Munns, 2014). The abnormal matrix, characterised by lack of cross-links, has been suggested to indirectly contribute to the impairment of osteoblast precursor differentiation (Fernandes et al., 2009), which correlates with an increase of osteoclast recruitment, resulting in further imbalanced bone turnover (Li et al., 2010; Forlino et al., 2011).

Recently, in a mouse model of the disease, it was demonstrated that transforming growth factor β (TGF-β) is present in excessive amounts in its active form in OI (Grafe et al., 2014a). TGF-β is secreted by osteoblasts and stored in the ECM due to its interaction with non-collagenous proteins such as the latency-associated peptide (LAP) and small leucine-rich proteoglycans (SLRPs), such as decorin (Hildebrand et al., 1994; Lim et al., 2017). Release of TGF-β from the ECM has been reported during bone resorption by osteoclasts, thus locally attracting bone-forming osteoblasts (Oreffo et al., 1989; Dallas et al., 2002). In a physiological setting, therefore, it appears that TGF-β plays an essential role in bone turnover. When present in excessive amounts, however, TGF-β signalling has been linked to the inhibition of osteoblast differentiation and the promotion of osteoclast activity, resulting in low bone density (Hattersley and Chambers, 1991; Erlebacher and Derynck, 1996; Alliston et al., 2001). In the study, *crtap*-/- mice were characterised by excessive TGF-β signalling and had features of the recessive form of OI including low bone mass and impaired osteoblasts. Interestingly, treatment with an inhibitor of TGF-β corrected most of the OI phenotype. Excessive TGF-β signalling in OI might be due to the abnormal ECM which is unable to correctly retain TGF-β in its latent form (Grafe et al., 2014b).

1.2.3 Classification

In the last decade, it has become evident that OI syndromes are not separate diseases and that the range of severities rather lies on a continuum. Nevertheless, the categorisation of patients into different types has proven effective to evaluate prognosis and assess the therapeutic benefits of treatments (Kamoun-Goldrat and Le Merrer, 2007). A first classification of the disease, proposed in 1979 and based on

radiographic and clinical features, divided the dominant OI types into 4 groups: type I as a mild form, type II as a perinatally lethal form, type III as the most severe survivable form of the disease, characterised by progressive deformity, and type IV as an intermediate form (Sillence and Rimoin, 1978; Sillence et al., 1979) (**Figure 3**). This classification, however, only considers those types with an autosomal dominant pattern of inheritance and are caused by mutations in *COL1A1* and *COL1A2* genes.

Following the discovery of different mutations leading to the disease, some of which following a recessive inheritance pattern, the classification was expanded to cover the whole spectrum and new types were added (**Table 1**). According to the most widely used classification of the disease, which categorises OI types according to the genetic mutation and resulting disease aetiology, there are 11 types of OI, including both dominant and recessive forms (Forlino et al., 2011). Type V and VI are caused by mutations in *IFITM5* and *SERPINF1* and have mineralisation defects, types VII, VIII and IX are very severe/lethal forms caused by mutations in genes involved in 3-hydroxylation (*CRTAP, LEPRE1* or *PPIB*), while types X and XI are severe forms caused by defects in chaperone proteins (SERPINH1 and FKBP10). This classification is being constantly expanded with the discovery of new mutations.

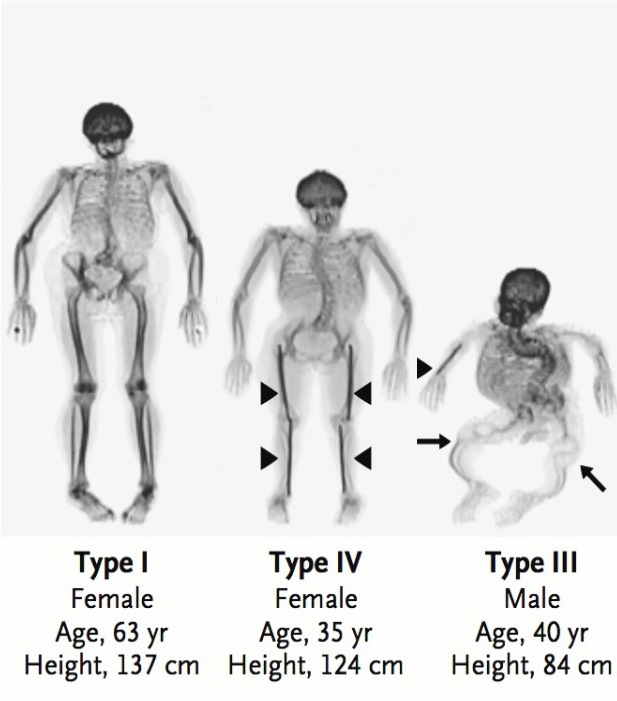


Figure 3. Types of OI

The four classic types of OI differ in their severity, ranging from the mild type I, to the intermediate type IV to the severe type III. Type II is perinatally lethal. Adapted from ***(Reeder and Orwoll, 2006)****.*

OI type	Mutated gene	Phenotype	Inheritance
I	*COL1A1*	Mild	Autosomal dominant
II	*COL1A1 / COL1A2*	Perinatally lethal	Autosomal dominant
III	*COL1A1 / COL1A2*	Severe	Autosomal dominant
IV	*COL1A1 / COL1A2*	Moderate	Autosomal dominant
V	*IFITM5*	Moderate	Autosomal dominant
VI	*SERPINF1*	Mineralisation defects	Autosomal recessive
VII	*CRTAP*	Severe	Autosomal recessive
VIII	*LEPRE1*	Severe / lethal	Autosomal recessive
IX	*PPIB*	Moderate / severe	Autosomal recessive
X	*SERPINH1*	Severe	Autosomal recessive
XI	*FKBP10*	Progressively deforming	Autosomal recessive

Table 1. OI classification.

Summary of the different OI types, inheritance patterns, phenotypes and underlying genetic mutations. Adapted from (Forlino et al., 2011).

1.2.4 Clinical management

Diagnosis and clinical management of OI are challenging. It is advisable for clinicians to run full screenings for OI-causative genes, even after a first mutation is found, in order to better address the complexity of each different case. However, due to the high cost of the test, a screening for mutations on *COL1A1* and *COL1A2* is usually performed first and full sequencing of the other genes is performed only in case of

negative results (Forlino and Marini, 2016). Clinical management is usually performed by a multidisciplinary team in a specialised centre with experience of OI and aims at promoting normal function, relieving pain and improving the quality of life.

Physiotherapy and hydrotherapy, aimed at strengthening the muscles and increasing the range of motion of joints, are highly recommended to improve independence (Brizola et al., 2014). Decline in pulmonary functions and lung infections are the most common causes of mortality in OI patients (Singer et al., 2001).

Monitoring of cardiac function is also essential, since aortic dilation and valvular defects, especially mitral regurgitation, are common complications in OI patients (Lamanna et al., 2013). Hearing assessment is also important, since early onset hearing loss happens in 5% of cases (Kuurila et al., 2000).

1.2.5 Pharmacological therapy

In terms of treatment, the use of bisphosphonates remains the gold standard pharmacological therapy for OI. Bisphosphonates are synthetic analogues of inorganic pyrophosphate that act as osteoclast inhibitors, inducing cell death (Rogers et al., 2000). They bind to the hydroxyapatite crystals of the bone, from which they are endocytosed by osteoclasts during bone resorption, causing apoptosis. Treatment is therefore aimed at increasing bone mass by inhibiting bone resorption. The rationale of this therapy is that more bone mass, even if still defective, improves bone strength (Forlino and Marini, 2016). Bisphosphonate treatment gives some therapeutic benefits to most children, with the highest results in the second and third year of administration. Bisphosphonates have a very long half-life and beneficial effects can last for years (Rauch et al., 2002). Despite being useful for many patients, bisphosphonates are unable to reduce the long-term incidence of fractures and to reduce bone pain (Ward et al., 2011). Moreover, recent meta-studies that considered several placebo-controlled randomised trials concluded that the data fail to demonstrate a statistically significant decrease in fracture rate and sustained bone pain relief in OI patients treated with bisphosphonates (Dwan et al., 2014; Hald et al., 2015). The inefficiency in reducing the number of fractures despite increasing bone

mass is possibly explained by the consequent increase in bone brittleness due to the higher mineralisation, as observed in mouse models (Uveges et al., 2009). Denosumab, a blocking antibody to RANKL, is another osteoclast inhibitor which has recently shown therapeutic effect on children with type IV OI, by decreasing the rate of bone resorption (Semler et al., 2012).

Other pharmacological options include the administration of growth hormone and human recombinant parathyroid hormone. Growth hormone shows anabolic effects but no data on long-term effects are available (Thomas and DiMeglio, 2016), while parathyroid hormone has proven promising in type I OI patients, in which bone mineral density was increased (Orwoll et al., 2014). Novel pharmacological therapies that are currently being tested on rodent models, include antibodies against sclerostin, a member of the Wnt pathway which inhibits bone formation (Bonewald, 2011; Sinder et al., 2013), and TGF-β, which is present in excessive amounts in OI (Grafe et al., 2014b).

1.2.6 Surgery

Corrective surgery, particularly osteotomy of long bones with the insertion of intramedullary telescopic rods, is often performed in order to allow ambulation and manage fractures, complemented with physical rehabilitation aimed at strengthening of muscles. Despite the great advances of surgery techniques and technologies, non-unions occur in about 15% of osteotomy procedures in OI and rods migration towards a joint has been reported (Luhmann et al., 1998). Due to the inability of these treatments to target the underlying molecular defect, other approaches have been more recently explored, particularly gene and cell therapy.

1.2.7 Gene therapy

Gene therapy for OI aims at correcting the causative mutation or replacing the defective gene, in order to achieve a long lasting therapeutic effect. In the recessive forms of OI, the deficiency of factors required for collagen processing and post-translational modifications could be reverted by introducing new copies of the gene.

On the other hand, dominant forms with structural defects of collagen would rather benefit from the silencing of the mutated gene. Antisense oligonucleotides, designed to target a specific mutation, have been investigated to suppress the expression of the mutant *COL1A2* gene, *in vitro*, in fibroblasts isolated from OI type IV patients. This approach was able to decrease the amount of mutant protein by 50%, however it also partially suppressed the normal allele (Wang and Marini, 1996). Despite the initial interest in this approach, in the past decades the scientific community has focused on other strategies. Collagen type I genes contain more than 50 exons each and more than 800 different OI-causing mutations have been identified on these two genes alone. This genetic heterogeneity makes it difficult and time consuming to design vectors for specific mutations (Niyibizi et al., 2004; Forlino et al., 2011). Adenoviral vectors have been successfully used to target mutations on the *COL1A1* and *COL1A2* genes in MSCs *in vitro* and *in vivo* in animal models, restoring the production of correct collagen fibres (Chamberlain et al., 2004; 2008).

1.2.8 Gene therapy with iPS and ES cells

One of the main drawbacks in the use of MSCs for gene therapy is the limited replicative capacity of adult MSCs, which cannot be expanded in high numbers needed for therapeutic applications (Zaim et al., 2012). In order to tackle this issue, several groups have developed protocols for the generation of pluripotent stem cell-derived MSCs, either from induced pluripotent stem cells (iMSCs) or from embryonic stem cells (eMSCs) (Frobel et al., 2014; Wang et al., 2014). To investigate the potential of iPS-based gene therapy for OI, iPS cells have been derived from previously gene-corrected MSCs from OI type III and IV patients, and subsequently differentiated *in vitro* into osteoblasts, able to form bone *in vivo* if transplanted to mice (Deyle et al., 2012). These promising approaches are still at an early stage and need to be further validated, particularly with respect to the possibility of random integration of viral vectors and of tumour formation in pluripotent stem cell-derived MSC transplantation.

1.3 Rationale for cell therapy with MSCs

Much interest in the field of regenerative medicine is focused on cell therapy, which promises to regenerate damaged tissues by providing healthy cells as replacements. Cell therapy is based on few key characteristics of stem cells, such as their ability to engraft into the damaged tissues, self-renew and differentiate into the appropriate cell type, by responding to the cellular niche, and at the same time modify such niche by secreting soluble factors (Nitkin and Bonfield, 2017). Different types of stem cells have been investigated as potential candidates for cell therapy in various disorders. In the case of OI, the most promising and widely studied cell source is represented by MSCs.

1.3.1 Mesenchymal stem/stromal cells

MSCs have been described and officially named in the early 1990s as adult cells, derived from the bone marrow, able to be expanded *in vitro* and to differentiate into cells of the different mesodermal lineages (Caplan, 1991; Haynesworth et al., 1992). The first reports of plastic-adherent progenitor clonal cells with osteogenic potential from the bone marrow, however, date much earlier (Friedenstein et al., 1968; 1970; Owen and Friedenstein, 1988). The widely accepted criteria to describe MSCs have been agreed by the International Society for Cellular Therapy in 2006. They define MSCs as a plastic-adherent, non-hematopoietic multipotent stem cells, able to differentiate into bone, cartilage and adipose cells. A panel of surface markers to define MSCs has also been described. MSCs are currently identified as cells positive for the surface markers CD73 (ecto-5'-nucleotidase), CD90 (Thy-1) and CD105 (endoglin) and lacking expression of the hematopoietic markers CD45 (protein tyrosine phosphatase, receptor type C pan-leukocyte marker), CD34 (hematopoietic progenitor cell antigen CD34) and CD14 monocyte marker (Dominici et al., 2006). Despite these attempts to precisely identify MSCs, the exact definition remains elusive, due to the high variability in the pattern of expression of MSC markers and in their ability to differentiate depending on the stem cell niche of the tissue of origin (Kuhn and Tuan, 2010).

It has been reported that MSCs reside in many adult and fetal tissues and protocols have been developed to isolate and culture MSCs from a variety of sources (Ullah et al., 2015; Pérez-Silos et al., 2016). In addition to the bone marrow, efficient protocols have been established to isolate adult MSCs from adipose tissue (Zuk et al., 2001; Wagner et al., 2005), dental tissue (Huang et al., 2009), menstrual blood (Allickson et al., 2011), skin (Bartsch et al., 2005) and synovial fluid (Morito et al., 2008).

The increased knowledge in the biology and immune-modulation of MSCs, together with the bulk of information derived from pre-clinical studies, have led to a boost in the translation of MSC-based cell therapy strategies to the clinics. In recent years, there has been a steady increase in the number of registered clinical trials using MSCs for a variety of conditions, including skeletal diseases, heart conditions, diabetes and autoimmune disorders among the most addressed (**Figure 4**).

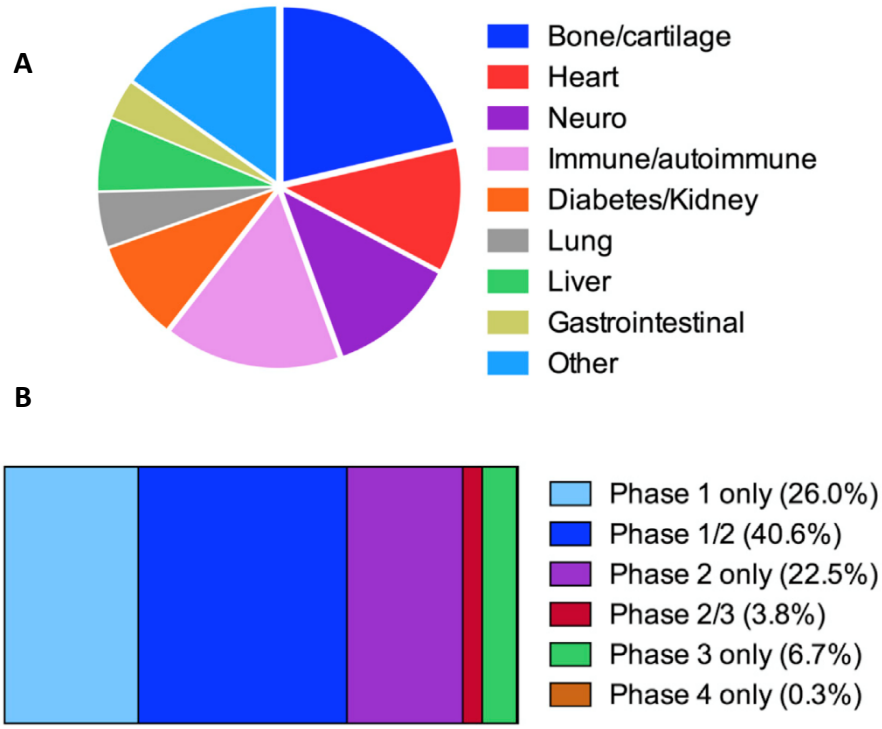


Figure 4. MSCs in clinical trials.

A) Diseases addressed by MSC transplantation in clinical trials (total of 352 trials). B) Clinical phase of MSC trials (total 315 trials). (Trounson and McDonald, 2015).

Adult bone marrow, adipose-derived MSCs and fetal MSCs of various origin are by far the most used cell types in clinical trials happening across the world at the moment. The NIH database lists more than 400 registered clinical trials involving MSCs, completed or ongoing, which is a 3-fold increase since 2011 (Trounson and McDonald, 2015). The large majority of these trials are however still in phase I or II. According to the Food and Drug Administration (FDA) guidelines, phase I trials aim at solely assessing safety and identifying the most common adverse effects of a therapy, while phase II trials are early investigations of therapy effectiveness. Very few trials involving MSCs are in the more advanced phase III, which compares the effectiveness and safety of the new therapy to the ones currently available, and IV, which gathers additional information on approved therapies (Squillaro et al., 2016).

1.3.2 Migration to site of injury

One of the key features of MSCs to be exploited in the development of cell therapies is their apparent ability to migrate towards the site of injury and engraft into the target tissue, where they exert therapeutic functions. MSCs have been shown to migrate towards specific stimuli, represented by the release of cytokines, chemokines and growth factors from the injured tissue (Fox et al., 2007). In a physiological context, the stem cell niche is a dynamic microenvironment constituted by secreted factors which provides cues to stem cells to maintain their stemness, proliferate or differentiate in response to local signals (Schofield, 1978). When injury occurs, the secretion of inflammatory cytokines acts as a stimulus for MSC trafficking and homing, by facilitating migration, adhesion and trans-endothelial movement (Chavakis et al., 2008).

As stem cell therapy capitalises on this natural occurring event to deliver donor cells directly to the site of injury, the understanding of the composition of this dynamic environment as well as the interplay between donor cells and target tissue is pivotal for the development and translation of cell therapies (Dimmeler et al., 2014). The mechanism directing leukocytes across the endothelium and towards areas of infection or injury has been well studied and is based on the expression of chemokine receptors on the cell surface, which respond to the stimulus of secreted inflammatory

cytokines released locally by damaged tissues, leading to cell migration (Miyasaka and Tanaka, 2004). In a similar manner, MSCs are rich in chemokine receptors and similar mechanisms involved in their migration have been investigated for their potential application in cell therapy. Studies have shown that MSCs undergo extravasation following a multi-step process similar to the one of leukocytes, involving initial contact with the endothelium, tethering and endothelial rolling (Henschler et al., 2008; Nitzsche et al., 2017).

Interestingly, it has been reported that MSCs home preferentially in cardiac and brain tissues following infarction or injury and into bones following fractures (Granero-Moltó et al., 2009; Ghadge et al., 2011). Injured tissues release a variety of soluble factors that act as a signal to MSCs, including stromal-derived factor-1 (SDF-1) and hepatocyte growth factor (Kucia et al., 2004; Son et al., 2006), bone morphogenetic proteins BMP2 and BMP4, platelet derived growth factor (PDGF-bb) and insulin-like growth factors IGF-I and IGF-II (Fiedler et al., 2002; 2006). At the same time, MSCs express a broad range of chemokine receptors including CCR1, CCR7, CCR9, CXCR4, CXCR5 and CXCR6 (Honczarenko et al., 2006).

SDF-1 is a particularly relevant chemokine for donor cell homing and has been object of extensive study. When SDF-1 is recognised by its receptor CXCR4, the interaction causes cytoskeletal reorganisations within the cell, leading to migration toward the SDF-1 gradient and therefore favouring trans-endothelial migration (Dar et al., 2006). The importance of SDF-1/CXCR4 interaction for donor cell homing is highlighted by studies that showed the inability of CXCR4-negative cells to home in the site of bone fracture and the increased homing of fetal MSCs following priming with SDF-1 (Granero-Moltó et al., 2009; Jones et al., 2012a).

Not all MSCs possess the same migratory ability, which strongly depends on the tissue of origin. In a study that compared the migratory capacity of MSCs from different origins, fetal MSCs derived from the lungs showed higher mobility compared to fetal bone marrow MSCs, which showed similar levels of migration to adult bone marrow MSCs. Also, within the same MSC population, only a small fraction of cells showed migratory abilities (Maijenburg et al., 2010). It has also been reported that prolonged

ex vivo culture of MSCs leads to changes in the expression of surface markers correlated to a diminished homing ability (Kim and Park, 2017). The careful selection of the appropriate MSC source and the evaluation of possible priming strategies appear of key importance during the design of cell therapy studies, in order to optimise donor stem cell homing in the target tissue.

1.3.3 Engraftment

One of the major challenges in cell therapy is the achievement of high numbers of donor stem cells in the target tissue that show long term engraftment and survival. Often injury and disease leave tissues highly damaged and a large number of donor cells are needed to replace a massive amount of dead or impaired endogenous cells. Poor engraftment remains one of the major hurdles in the development and translation of all stem cell therapies, including those with established clinical protocols such as hematopoietic stem cell transplantations (Dimmeler et al., 2014).

Different studies of cell-tracking in rodent models transplanted with MSCs showed very low engraftment levels, ranging from 2 to 10% in the first weeks and almost none after 3 months (Gu et al., 2012; Dimmeler et al., 2014). On the other hand, the short lifespan and limited replicative capacity of MSCs may guarantee safety form malignant transformation following transplantation (Kim and Park, 2017). The only cell type that has shown long-term engraftment and survival is embryonic stem cells in their undifferentiated state, which however formed tumours when transplanted (Gu et al., 2012).

A possible explanation for the short lifespan of transplanted cells is found in the hostile environment that cells encounter when reaching the damaged tissue. Injured tissues are characterised by high concentrations of inflammatory cytokines and low levels of oxygen. Another possible explanation is that often donor cells are transplanted into mature tissues that appear to be less permissive than developing tissues to donor cell integration. For this reason, more recently, *in utero* transplantation of stem cells has been investigated for a variety of conditions, including osteogenesis imperfecta (Guillot et al., 2008). Intervening early in

development, with either *in utero* or neonatal transplantation, would allow to address the defect before irreversible tissue damage occurs. This approach benefits from stoichiometric advantage, since fewer cells are needed to treat a fetus or a neonate than an adult.

The levels of engraftment also depend on the route of administration used for the delivery of stem cells. The most widely used route is intravenous delivery which allows the infusion of large number of cells. It is however known that large numbers of MSCs delivered in this way are unable to reach the target tissue and remain trapped in the lungs, due to the larger size of cells compared to the pulmonary vasculature (Fischer et al., 2009). More recently, local MSC delivery through a scaffold has been investigated to repair bone defects, achieving lager engraftment (Nitkin and Bonfield, 2017).

1.3.4 Differentiation

The ability to differentiate into cells of different mesodermal lineages with high efficiency is crucial for the use of MSCs for regenerative medicine purposes. The classic rationale behind stem cell therapies is based on the idea that MSC transplantation will benefit tissue repair due to the ability of these cells to differentiate into specific cells of the target tissue and repopulate the damaged organ, by replacing deficient resident cells (Squillaro et al., 2016). MSCs of various origin are able to differentiate efficiently into cells of the adipogenic, chondrogenic and osteogenic lineages. More recently it has been reported that MSCs isolated form fetal tissues have a broader differentiation potential as they are able to differentiate into limited cell types of ectodermal and endodermal origin as well (De Coppi et al., 2007; Abdulrazzak et al., 2010).

In a cell therapy approach, stem cells can be transplanted either in their naïve, undifferentiated state, or in a partially or fully differentiated state. While naïve stem cells have a higher replicative life span, differentiated cells may be better able to respond to the niche of the target tissue and to produce the appropriate cytokines and growth factors that are needed to repair the injured tissue (Nitkin and Bonfield,

2017). Moreover, the differentiation ability of stem cells greatly depends on the tissue of origin and it is well reported that differentiation potential narrows with increasing donor age (Choudhery et al., 2014). It has for example been reported that MSCs shift their differentiation commitment from the osteogenic to the adipogenic lineages with increasing cellular senescence (Kim et al., 2012).

1.3.5 Paracrine effect

In addition to directly replacing damaged cells, stem cells appear to be also able to repair a tissue by acting in a paracrine manner. A growing body of evidence shows that MSCs secrete a broad variety of soluble factors, including cytokines, chemokines and growth factors, which is commonly referred to as "MSC secretome" (Doorn et al., 2012). Moreover, MSCs release large numbers of extracellular vesicles, membrane-derived bodies containing peptides, lipids and microRNAs that are involved in cell-to-cell communication (Rani et al., 2015).

The trophic effect of MSCs has recently become of great interest in the field of regenerative medicine, since it could explain why therapeutic benefits are observed following transplantation of MSCs, even when levels of engraftment are very low (Iso et al., 2007; Wang et al., 2011; Phinney et al., 2015; Prockop, 2017). It is now widely accepted that paracrine factors, locally secreted by MSCs, modify the microenvironment and indirectly promote tissue regeneration by stimulating endogenous cell proliferation and preventing apoptosis of resident cells (Caplan and Dennis, 2006). This role is exemplified also by the function that MSCs exert in a physiological setting. MSCs are found in a wide variety of tissues, where they act as stromal support to other cells of the hematopoietic and endothelial niche (Quesenberry et al., 1989; Spees et al., 2016).

More recently, however, a new paradigm for the paracrine effect of MSCs has taken shape. MSCs appear to be able to influence the fate of cells that are very distant from the site of original engraftment, possibly through the release of soluble factors and extracellular vesicles that travel through the vasculature. This new model could explain the remarkable healing properties of MSCs in the numerous cases where cells

remain trapped in the lung microvasculature following transplantation, due to their size (Spees et al., 2016). Many studies describe beneficial effects of MSC-derived conditioned medium in a variety of pathological settings. Some of the most convincing evidence comes from studies on rodent models of myocardial and kidney injuries. Transplantation of MSCs did not result in long-term engraftment but therapeutic benefits were observed in both cases, which were explained by the secretion of trophic factors (Iso et al., 2014). The same effects were indeed obtained after administration of MSC-derived conditioned medium (Bi et al., 2007).

Despite extensive research, the exact mechanisms that govern the paracrine effects of MSCs in the context of tissue repair remain elusive. Nevertheless, the idea of being able to inject cell-free factors instead of cell preparations is highly appealing, since this strategy would avoid any adverse effects and rule out any possibility of immune rejection and tumour formation.

1.3.6 Safety

One of the main hurdles of cell therapy strategies is the immune rejection of transplanted cells in an allogeneic setting. The privileged immunologic properties of MSCs became evident for the first time from *in utero* transplantation of MSCs conducted on sheep. Cells were not rejected and were able to engraft, even after immune competence of the fetus was acquired (Liechty et al., 2000). Surprisingly, no significant difference was observed in terms of therapeutic efficacy between autologous and allogeneic MSCs, indicating that MSCs are a remarkably safe source of cells for cell therapy. MSCs have been used as a cell source for transplantations for more than 15 years in thousands of patients and it is a general consensus that they are clinically safe (Trounson and McDonald, 2015).

The absence of immune response against transplanted MSCs is mainly due to the lack of expression of human leukocyte antigen (HLA) class II and low levels of expression of HLA class I, which protects MSCs from being lysed by natural killer cells (Rasmusson et al., 2003; Giuliani et al., 2014). MSCs can only differentiate into cells of mesodermal lineages and there have been no reports of malignant transformation or formation of

teratomas *in vivo* (Götherström, 2016). Moreover, the transient nature of MSCs, which only have a limited lifespan in the host following transplantation, is a further guarantee of safety against malignant transformation (Trounson and McDonald, 2015; Kim and Park, 2017).

1.3.7 Immunomodulation

The unique immunomodulatory properties of MSCs have been of great interest in the field of regenerative medicine in the last two decades. A bulk of evidence from both *in vitro* and *in vivo* studies have demonstrated that MSCs interact with a variety of immune cells. MSCs have been shown to be able to directly inhibit the proliferation of natural killer and cytotoxic T cells (Le Blanc et al., 2003; Selmani et al., 2008). Moreover, MSCs increase regulatory T cells (Tregs), thus indirectly decreasing in the activity of cytotoxic T cells (Glenn and Whartenby, 2014). The immunosuppressive activity of MSCs has been reported to be stimulated by T-lymphocyte-produced cytokines such as tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ), which causes the production of nitric oxide, indolamine-2,3-dioxygenase (IDO) and prostaglandin (DelaRosa et al., 2009; Ghannam et al., 2010). On the other hand, reports have shown that MSCs may also stimulate the immune system and recruit immune cells if the level of pro-inflammatory cytokines is too low, acting as sensors of inflammation (Bernardo and Fibbe, 2013).

Due to their suppressive effects on the immune system, MSCs have been investigated in a clinical setting as a possible treatment for acute cases of graft versus host disease (GVHD), a severe consequence to transplantation of allogeneic grafts in which the host immune system recognises and attacks the graft. Phase II clinical trials for steroid-resistant GVHD with repeated infusions of bone marrow MSCs have shown beneficial effects with half of the patients responding to the therapy and having a higher 2-year survival rate compared to non-responders (53% vs. 16%) (Le Blanc et al., 2008; Prasad et al., 2011). Due to their low immunogenic profile and immune modulatory properties, MSCs are widely considered a promising source of donor cells for cell therapy and regenerative medicine.

1.3.8 Limitations

Despite the successes of the recent years, many limitations in the translation of cell therapy strategies remain. MSCs are very heterogeneous and vary greatly according to the tissue of origin, age of donor and culture conditions. Therefore, more standardised protocols are needed for the further development of cell therapies and to be able to compare results. Moreover, MSCs are usually subject to prolonged expansion, since high number of cells are needed for transplantation. Prolonged *ex vivo* culture has been linked to genomic variations and altered cell phenotype (Bara et al., 2014). MSCs only have a limited replicative life-span and MSCs derived from older donors might reach cellular senescence, thus impairing their homing, differentiation and immunomodulatory potential (Kim and Park, 2017). Due to this issue, different MSC sources with higher proliferative potentials have been investigated, including fetal MSCs of various origins.

1.4 Fetal sources of mesenchymal stem cells

The use of MSCs from adult tissues for regenerative medicine purposes is limited due to the limited proliferation capacity of cells, which reach senescence. To overcome this problem, MSCs derived from perinatal and fetal tissues have attracted the attention of the scientific community as promising alternative to adult MSCs for cell therapy and tissue engineering applications.

1.4.1 MSCs in development

MSCs appear in the fetal blood from early gestation, before migrating to the bone marrow. The most widely accepted hypothesis is that MSCs provide stromal support to HSCs, as shown by studies in mouse models in which MSCs favoured the engraftment of HSCs (Anklesaria et al., 1987; Noort et al., 2002). MSCs represent around 0.4% of all nucleated fetal blood cells during first trimester and their number dramatically declines with age (Gucciardo et al., 2009). In the fetus, the ratio of MSC / total nucleated cells in the bone marrow is 1:400, which is reduced to 1:10,000 in newborns and to 1:250,000 in adults (Caplan, 1994; Campagnoli et al., 2001; Götherström, 2016).

1.4.2 Advantages and limitations of fetal MSCs

Fetal MSCs share the same phenotype, secretory profile and low immunogenic properties as their adult counterparts and present additional advantages. They are more proliferative, having an average doubling time of 30 hours compared to the 80 hours of adult MSCs and achieve an average of 70 population doublings without reaching senescence, compared to the 15-40 of adult MSCs (Guillot et al., 2007; Götherström, 2016). Fetal MSCs, moreover, have longer telomeres than MSCs derived from adult tissues, due to the presence of active telomerase (Guillot et al., 2007; Bieback and Brinkmann, 2010). Notwithstanding their higher proliferative capacity, fetal MSCs are non-tumorigenic as evidenced by their inability to form teratomas when transplanted (Guillot et al., 2006). MSCs from fetal tissues also present a more primitive phenotype, expressing some pluripotency markers and

being able to differentiate into more cell types, such as muscle and neurons, than adult MSCs (Chan et al., 2007; Kennea et al., 2009). Finally, MSCs from fetal tissues have been reported to have a higher osteogenic potential than their adult counterparts (Zhang et al., 2009). Due to the favourable properties of fetal cells, numerous protocols have been established to isolate MSCs from a variety of fetal and perinatal tissues, including bone marrow, liver, blood and extra-embryonic tissues such as the placenta and the amniotic fluid.

The collection of cells from tissues of the fetus implies the termination of pregnancy. The only exception is cardiocentesis that can be performed on live fetuses for the isolation of fetal blood stem cells. It however carries a high miscarriage risk and high morbidity for the mother and it cannot be performed routinely (Chan et al., 2008; Sarno and Wilson, 2008). Moreover, since most terminations are carried out in the early stages of gestation, it is usually only possible to isolate small number of cells, due to the small size of the fetus. Finally, although such fetal material would be discarded following a termination, ethical restrictions limit the clinical use of fetal MSCs (Cananzi et al., 2009). Due to these limitations, different cell sources that are ethically available, safe to collect and can yield a high number of cells are needed to further investigate and eventually translate fetal cell therapy into clinical practice.

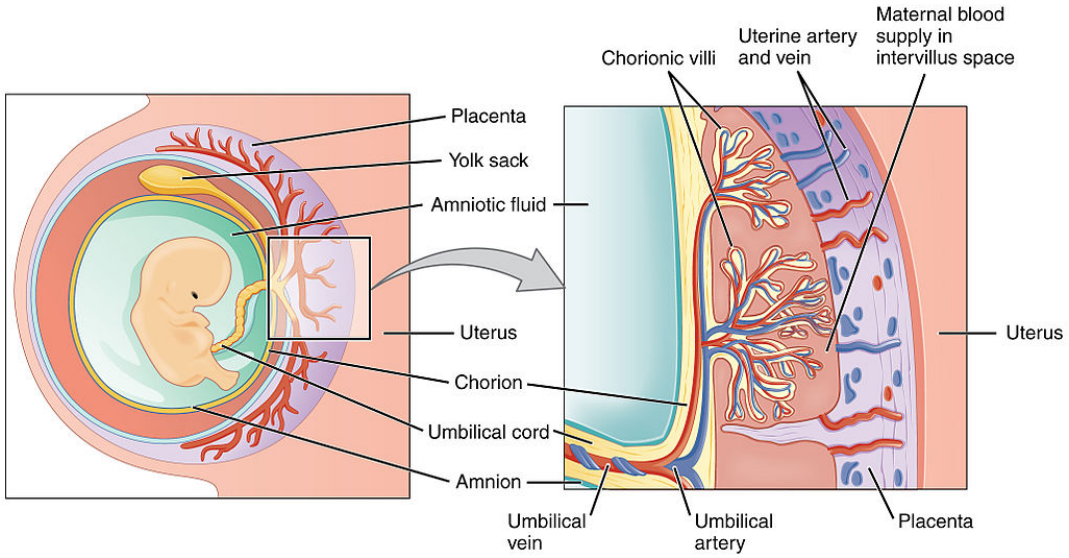


Figure 5. Extraembryonic tissues.

Fetal MSCs can be isolated from a variety of extraembryonic tissues, including placenta, umbilical cord and amniotic fluid (Anatomy & Physiology, 2016, Rice University).

1.4.3 Extra-embryonic tissues

The extra-embryonic tissues are rich in fetal stem cells. They can be easily collected at delivery or during invasive prenatal diagnostic procedures that are now routinely used in clinics. Cells isolated from such tissues represent a valid and ethical alternative source of stem cells for regenerative medicine applications, including cell and gene therapy as well as tissue engineering (Loukogeorgakis and De Coppi, 2016). **Figure 5** depicts the extra-embryonic tissues that surround the fetus during pregnancy.

1.4.4 Placenta

The placenta is an organ which develops during pregnancy to nourish and sustain the development of the fetus, by supporting the transfer of oxygen, nutrients and waste products between the mother and the fetus. Recently, the placenta has been identified as a rich source of fetal stem cells. Placental tissue can be isolated during chorionic villous sampling, an invasive prenatal diagnosis, from the 10^{th} week of gestation (Ilancheran et al., 2009) and during delivery, when it is usually discarded (Antoniadou and David, 2016). Chorionic mesenchymal stem cells have been reported to possess a more primitive phenotype and a faster growth kinetics if compared to adult MSCs (Soncini et al., 2007; Barlow et al., 2008; Jones et al., 2012b). They express the pluripotency markers OCT4 and SSEA-3 as wells as the MSC markers and are able to differentiate in cells of the mesodermal lineage (Battula et al., 2007; Poloni et al., 2008). It has also been reported that placental MSCs can be differentiated into cells of the hepatic and neurogenic lineages (Portmann-Lanz et al., 2010; Kim et al., 2011). Gestational age greatly influences the properties of placental MSCs; cells isolated at younger developmental ages have a higher proliferation potential and differentiation ability than cells isolated at the end of pregnancy (Sung et al., 2010; Park et al., 2013).

As other fetal MSCs, placental stem cells have low immunogenicity, due to the low expression of HLA molecules and also possess immunomodulatory properties (Antoniadou and David, 2016). Placental MSCs are able to suppress the proliferation

of T-lymphocytes and have been shown to shift the macrophage phenotype from inflammatory to anti-inflammatory (Poloni et al., 2012; Abumaree et al., 2013). The main hurdle for the isolation of placental MSCs is the contamination with decidual maternal cells (In 't Anker et al., 2004; Soncini et al., 2007).

1.4.5 Umbilical cord

The umbilical cord is a flexible structure connecting the developing fetus to the placenta. It contains two arteries and a vein, surrounded by mucus-rich connective tissue and covered by an amnion-derived epithelium. The umbilical cord is another rich source of fetal MSCs. They can be easily isolated at delivery from the connective tissue which forms the largest component of the umbilical cord, also called Wharton's jelly (McElreavey et al., 1991; Wang et al., 2004). In particular, fetal MSCs have been isolated from the perivascular and sub-amniotic areas of the Wharton's jelly (Karahuseyinoglu et al., 2007). Wharton's jelly MSCs express mesenchymal markers CD73, CD90, CD105 and CD146 and have been described as more primitive than adult MSCs in terms of faster proliferation kinetics and broader differentiation potential (Joerger-Messerli et al., 2016). Wharton's jelly MSCs can differentiate into the osteogenic, adipogenic and chrondrogenic lineages as well as into hepatocytes and neurons (Mitchell et al., 2003; Wang et al., 2004; Anzalone et al., 2010). In a comparative study, MSCs from the Wharton's jelly expressed lower levels of HLA II than bone marrow and adipose derived MSCs and showed a strong ability to inhibit T cell proliferation (Li et al., 2014)

1.4.6 Amniotic fluid

The amniotic fluid is a liquid found in the amniotic sac, which surrounds the fetus during its development. While consisting for 98% of water and electrolytes, it also contains hormones, nutrients, growth factors and cells of fetal origin (Cananzi et al., 2009). The main functions of the amniotic fluid are to protect the growing fetus against external traumas, while allowing it to move freely, and to act as a carrier of nutrients and water between the mother and the fetus (Underwood et al., 2005). The volume and content of the amniotic fluid varies greatly during fetal development. It

first starts developing around the 2nd week of pregnancy, when a small sac of fluid forms between the epiblast, which will then form the embryo, and the amnioblast, which will form the amnion membrane (Miki and Strom, 2006). By the 4th week of pregnancy, the amniotic fluid completely envelops the embryo. Initially the amniotic sac is entirely composed by water and solutes derived from maternal plasma, which pass through the fetal side due to osmotic and hydrostatic forces, following active transport of chlorine and sodium across the fetal membranes (Creasy et al., 2004). In the second half of the pregnancy, however, the fluid also contains fetal urine and excretions of the respiratory and digestive systems (Fauza, 2004). The volume of amniotic fluid increases considerably during pregnancy, starting from 20 ml at week 7 to 600 ml at week 25 reaching 1 litre at week 34 (Brace and Wolf, 1989).

The amniotic fluid contains fetal cells, the origin of which is still debated. The main hypothesis is that fetal cells from the skin, respiratory, urinary and gastrointestinal systems are shed into the fluid during fetal development (Fauza, 2004). The number of cells in the amniotic fluid increases with time during pregnancy, and it has been shown to further increase in case of spina bifida or anenchephaly, and abnormally decrease in case of urogenital atresia (Gosden and Brock, 1978). Different cell types have been identified in the amniotic fluid, initially according to their morphology. The first report describes viable adherent cells as amniocytes (61%), epithelial-like (34%) and fibroblast-like (5%) cells (Hoehn et al., 1975). More recently, progenitor cells with high proliferative and differentiation abilities were isolated from the amniotic fluid, both from the hematopoietic and the mesenchymal lineage (Torricelli et al., 1993; Streubel et al., 1996).

Due to their potential applications in regenerative medicine, MSCs from the amniotic fluid have generated a great interest and many protocols have been developed for their isolation and characterisation. Amniotic fluid can be safely collected by amniocentesis during the second trimester, when performing amnioreduction during the third trimester, or at caesarean section (Loukogeorgakis and De Coppi, 2017). From amniotic fluid samples, MSCs have been initially isolated by plastic adherence in high serum culture medium (Tsai et al., 2004). Subsequently, C-KIT (CD117, type III tyrosine kinase receptor for stem cell factor) has been used to select and isolate stem

cells in the amniotic fluid, which represent around 1% of the total amniotic cells (De Coppi et al., 2007).

Amniotic fluid stem cells have all the characteristics of fetal MSCs and also possess a broader differentiation potential, being able to also differentiate into cells of non-mesodermal lineages, including endothelia, hepatic and neuronal cells (De Coppi et al., 2007). The cellular characteristics of amniotic fluid MSCs vary according to the gestational age. Cells isolated from first trimester have a more primitive phenotype, sharing the majority of the transcriptome with embryonic stem cells, while second and third trimester cells have more mesodermal-committed phenotype (Moschidou et al., 2013a; Loukogeorgakis and De Coppi, 2017). Despite being able to differentiate into cells of the three lineages, amniotic fluid stem cells are unable to form teratomas *in vivo* when injected into immunodeficient mice (Roubelakis et al., 2007). This suggests that amniotic fluid stem cells are a safe cell source for regenerative medicine applications, although further studies with longer cultures are needed (Loukogeorgakis and De Coppi, 2017).

Analysis of the secretome of amniotic fluid MSCs showed the release of numerous growth factors and cytokines, including vascular endothelial growth factor (VEGF), endothelial growth factor (EGF), interleukin 6 (IL-6), and stromal cell-derived factor 1 (SDF-1) (Mirabella et al., 2011). Moreover, amniotic fluid MSCs possess strong immunomodulatory properties. Treatment of amniotic fluid stem cells with TNFα and INF-γ led to the inhibition of T-cells proliferation, both by direct cell contact and by conditioned medium, supporting the idea of a paracrine effect of MSCs (Kode et al., 2009; Joerger-Messerli et al., 2016).

1.5 Pre-clinical studies of cell therapy for OI

1.5.1 Mouse models of OI

Several mouse models of OI have been developed, both for the dominant and for the recessive forms of the disease. Here are listed three of the most commonly used.

Mov-13 mouse model of OI type I: the first model of OI was developed in the late 1980s by retroviral insertion of the Moloney murine leukaemia virus at the 5' end of the col1α1 gene encoding for the α1 chain of collagen type 1 (Harbers et al., 1984). This leads to the inhibition of the transcription of the gene and the absence of α1 chains in the model. The α2 chain is normally produced, however it is subject to degradation due to its inability to correctly fold in trimeric fibres. Homozygous mice do not produce any collagen type I and are not viable, whereas heterozygous mice produce around half of the physiological levels of collagen type I and have a normal lifespan and mild phenotype. Mov-13 mice manifest reduced mechanical properties of the long bones, increased bone porosity and progressive loss of hearing (Bonadio et al., 1990).

Oim/oim mouse model of OI type III: the most widely used model for pre-clinical studies on severe OI is the *oim/oim* mouse (osteogenesis imperfecta mouse), a model of human type III OI. Homozygous *oim/oim* mice (B6C3Fe a/a-Col1α2^{oim}/Col1α2^{oim}) are characterised by smaller size with short and thin bones, which are subject to multiple fractures, decreased bone strength and skeletal deformities (**Figure 6**). Heterozygous *oim/+* mice display only mild skeletal features, while homozygous oim/oim mice have substantial deformities (Saban et al., 1996). *Oim* mice live a normal lifespan. This phenotype is caused by a naturally occurring glycine deletion at nucleotide 3983 on the Col1α2 gene, encoding for the α2 chain of collagen type 1. The mutation causes a frame shift at the 3' end of the transcript, involving the sequence corresponding to the last 48 amino acids, and the subsequent production of altered collagen pro-peptides, that fail to assemble in the correct heterotrimeric COL1$(\alpha1)_2(\alpha2)_1$ fibre. As a consequence, abnormal homotrimeric collagen fibres, formed by three α1 chains, accumulate in the extracellular matrix (Chipman et al.,

1993). In homozygous *oim/oim* bones, the mineral component is abnormal and characterised by smaller and disorganised hydroxyapatite crystals, while the collagen component is less resistant to tensile stress (Misof et al., 1997). Taken together, these abnormalities lead to brittle and fragile bones. At the cellular level, moreover, *oim* osteoblasts fail to fully differentiate and remain in an immature stage, which leads to an increase in osteoclastogenesis, thus contributing to the abnormal bone turnover (Li et al., 2010b). In addition to the skeletal systems, other organs are affected; the production of abnormal collagen also alters myocardial mechanics (Weis et al., 2000), causes structural defects of the thoracic aorta (Pfeiffer et al., 2005) and leads to nephropathy (Phillips et al., 2002).

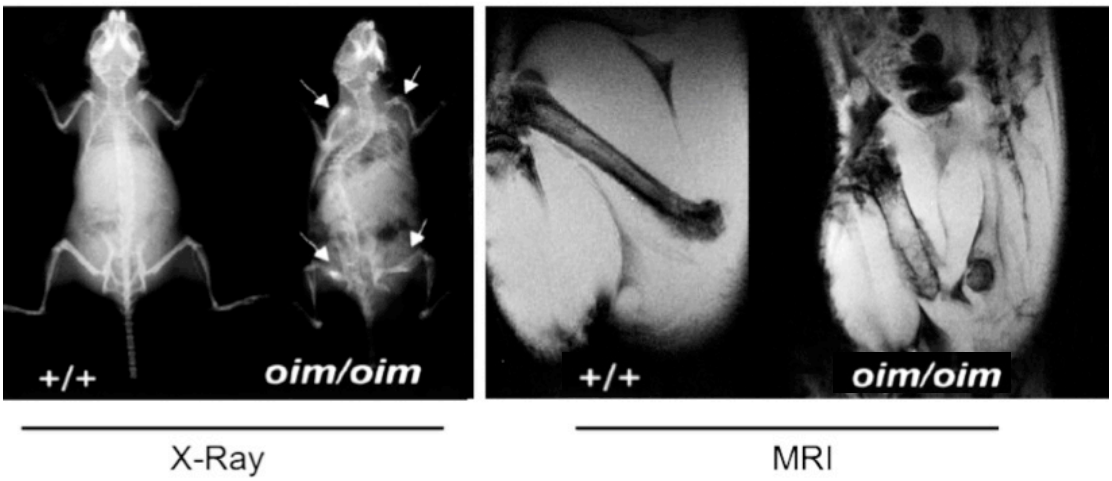


Figure 6. The oim model.

X-ray image, adapted from (Camacho et al., 2001) *and MRI scan (Courtesy of Dr. Pascale Guillot, UCL Institute for Women's Health) showing the skeletal deformities of oim mice compared to wild-type mice.*

Brtl IV mouse model of OI type IV: another widely used model is the Brtl IV (Brittle mouse), which has a glycine to cysteine knock-in mutation at position 349 of the *Col1α2* gene, obtained by cre-lox recombination. The Brtl IV model shares the same features as human type IV OI and manifests in a highly variable manner, with phenotypes ranging from perinatal lethality to normal lifespan with mild skeletal

defects (Forlino et al., 1999). Interestingly, heterozygous Brtl IV mice present severe reduction of bone mineral density and frequent fractures, while homozygote mice have a milder phenotype with normal mineral density, and moderately smaller size. This is probably because the homozygosity leads to a more homogeneous and therefore less brittle organisation of the extracellular matrix. As observed in type IV OI patients, the number of fractures in the Brtl IV mouse decreases with age. Moreover, it has been reported that the distinct phenotypes observed in the Brtl IV model result from the same ratios of mutant and normal collagen chains, therefore suggesting that interactions with extra-collagenous factors are critical for the different manifestation of the disease (Kamoun-Goldrat and Le Merrer, 2007).

COL1A1 minigene mouse model of OI type II-IV: the transgenic OI mouse model was obtained by insertion, through genome engineering techniques, of a mini-gene version of human *proα(I)* collagen gene characterised by normal promoter and 3' end region but lacking the central sequence, containing 41 exons (Stacey et al., 1988). The phenotype of the resulting mice was highly heterogeneous, with some mice displaying skeletal deformities, albeit without fractures. The human shortened proα(I) chains assemble with the endogenous proα(I) chains, leading to their degradation due to structural instability. The mini-gene OI model can be modulated to obtain a wide range of severities, from moderate type IV OI to severe/perinatally lethal type II OI, by increasing the level of expression of the human *proα(I)* mini-gene (Khillan et al., 1991). This transgenic line is particularly relevant because it has been the first to be used as a model of OI to assess the outcome of a cell therapy studies for OI, in 1998 (Pereira et al., 1998).

Several other mouse models have been developed to mimic the wide variety of genetic defects and resulting phenotypes that characterise the OI spectrum. **Table 2** summarises the main OI mouse models developed to date.

Model	Mutation	OI type	Reference
Mov13 $^{+/-}$ - Mov13 $^{-/-}$	M-MuLV insertion	I / II	Bonadio, 1990
Sp7 $^{-/-}$	Knock-out Sp7	II	Baek, 2009
Oim $^{-/-}$	Col1a2	III	Chipman, 1993
Aga2 $^{+/-}$	Col1a1	III	Lisse, 2008
Brtl $^{+/-}$ - Brtl $^{-/-}$	COL1A1 knock-in	IV	Forlino, 1999
Col1a1-minigene	Human COL1A1 knock-in	II / IV	Khillan, 1991
Col1a1 $^{jrt/+}$	Col1a1	IV / Ehles-Danlos syndrome	Chen, 2014
IFITM5 - transgenic	IFITM5	V	Lietman, 2015
Crtap $^{-/-}$	Knock-out Crtap	VII	Morello, 2006
Lepre $^{-/-}$	Knock-out Lepre	VIII	Vranka, 2010
Ppib $^{-/-}$	Knock-out Ppib	IX	Choi, 2009

Table 2. Mouse models of OI.

Summary of the mouse models developed for the study of OI. Adapted from (Jones and Guillot, 2013)

1.5.2 Cell therapy in OI animal models

Cell therapy has been proposed as a promising option for OI following observation of natural occurring mosaicism in OI families. OI mosaic carriers have a mixture of mutant and normal cell populations in all tissues, due to post-zygotic events, and only show minimal symptoms or are unaffected at all, even when a high proportion of their osteoblasts harbours the mutation (Dalgleish, 1998; Cabral and Marini, 2004). The original rationale of cell therapy for OI was therefore to mimic the mosaic situation, providing healthy osteoblast precursors that would be sufficient to normalise the disease phenotype, even with low engraftment (Gerson, 1999). The most relevant studies in OI mouse models to date are summarised in **Table 3**.

First studies: The first stem cell transplantation study was performed by injecting MSCs derived from the bone marrow of wild-type mice into a transgenic mouse model of OI, expressing the human minigene for COL1A1. 3-week-old transgenic mice where marrow-ablated by irradiation and transplanted intraperitoneally with MSCs ($0.7 – 12 \times 10^6$ cells per animal). Donor cell DNA was detected in the bone marrow, cartilage and lungs after 2.5 months following transplantation, giving the first evidence of engraftment in an OI model. Engraftment levels varied from 4 to 19%. Higher levels of collagen and minerals were detected in bones following transplantation, however no mechanical tests were performed on bones and no *in vivo* differentiation of donor cells was reported (Pereira et al., 1998).

Ten years later, two studies from Niyibizi's group confirmed the results of the previous study and were able for the first to show *in vivo* differentiation of donor cells in an OI model. In one study, 8-week-old wild-type mouse MSCs were labelled with green fluorescent protein (GFP) and injected into wild-type neonate mice via the temporal vein. After 35 days, those donor cells that had engrafted into the bones were retrieved. Analyses of the GFP^+ retrieved cells showed that they underwent partial osteogenic differentiation *in vivo*. These osteoprogenitors were then re-transplanted into previously irradiated neonate *oim/oim* mice, again via the temporal vein. Four weeks later, GFP^+ osteoblasts were detected in the epiphyses of long

bones, site of active bone formation, and in the cortex, showing further *in vivo* differentiation and contribution to bone formation (Wang et al., 2006).

The second study from the same group transplanted 5 x 10^4 GFP-labeled MSCs from wild-type mice into previously irradiated *oim/oim* neonates and were able to show engraftment into the bones. They observed for the first time that the collagen synthesized following transplantation was composed by both α1 and α2 chains that formed normal heterotrimeric collagen. Since the *oim* model is unable to produce Col1α2 chains, this was the first evidence that transplanted cells contributed to ECM formation *in vivo* (Li et al., 2007).

Intrauterine transplantations: The basis for *in utero* transplantation for the treatment of OI were suggested for the first time by a study performed injecting human first-trimester blood MSCs into *oim/oim* fetuses. MSCs were transduced with luciferase-expressing construct and intraperitoneally injected into the fetuses (10^6 cells per animal) following laparotomy. No bone marrow ablation or immune suppression was performed and human cells were not immune rejected following transplantation in mice. After 12 weeks, donor cells were shown to engraft at low level (5%) in bones and to differentiate into osteoblasts *in vivo*. Following transplantation, fracture rate was decreased by two thirds and bone mechanical properties were significantly improved (Guillot et al., 2008a).

A second intrauterine transplantation study from Guillot's group using human fetal blood MSCs confirmed the previous result, with an engraftment level of 5% and a 84% decrease in fracture rate, and showed that also donor human cells were able to produce the normal COL1A2 chain, missing in the *oim* model, and to improve the quality and stiffness of the ECM (Vanleene et al., 2011).

A similar experiment supporting intrauterine cell therapy was performed with the Brtl IV mouse model of OI. Bone marrow MSCs derived from GFP-transgenic mice were transplanted *in utero* into heterozygous Brtl IV mice (5 x10^6 cells per fetus). Donor cells were found to engraft and differentiate, contributing to 20% of the total amount of collagen produced. Brtl IV mice have normally a much shorter life span than wild-type mice. Following MSCs administration, transplanted mice lived twice longer than

non-transplanted controls and the mechanical properties of the analysed bones were improved (Panaroni et al., 2009).

Priming MSCs*:* In order to increase engraftment levels, Guillot's group later investigated priming human fetal blood MSCs with SDF-1 before intraperitoneally injecting them into oim/oim and wild-type neonate mice (10^6 cells/animal). Primed cells showed overexpression of CXCR4, which was related to improved chemotaxis and overall better engraftment. Higher engraftment levels positively correlated with a decrease in long bone fractures, although bone strength remained unchanged (Jones et al., 2012a).

Intra-femoral transplantation: Another study from Niyibizi's group proposed direct intra-femur administration of MSCs, to avoid large number of cells being trapped into the lungs. Mouse GFP-labelled MSCs were infused into the femurs of irradiated *oim/oim* mice (10^6 cells/animal), together with a collagen gel, to improve engraftment. After two weeks, new bone formation and increased mechanical properties were demonstrated in the femurs of transplanted mice and GFP^+ cells were detected 6 weeks after transplantation (Li et al., 2010a).

Another group tried a similar approach and injected MSCs from GFP-transgenic mice into the intramedullary cavity of the femur of previously irradiated *oim/oim* mice (10^6 cells/animal). Donor cells underwent osteogenic differentiation and new bone formation was observed. GFP^+ cells were then retrieved from the bones and re-transplanted into other oim/oim mice, to show the persistence of stem/progenitor cells *in vivo* (Pauley et al., 2014).

Ethical sources of MSCs: given the robust body of evidence supporting cell therapy for OI, both before and after birth, systemically and locally, the interest of the scientific community is now focused on the identification of the best stem cell source for the further clinical translation. Human fetal MSCs are not easily isolated and their use is ethically restricted since their availability relies on terminations of pregnancy. Recently, extra-embryonic fetal tissues have been investigated as possible sources of donor cells. In a recent study from Guillot's group, *oim/oim* neonate mice were intraperitoneally injected with human fetal early placental chorionic MSCs (10^6

cells/animal). Cells engrafted at low levels and differentiated into osteoblasts *in vivo.* Fracture rate decreased by two thirds and bone volume increased in transplanted mice, although bone strength was not improved. This was concomitant with an up-regulation of endogenous chondrogenic genes, suggesting a paracrine effect of transplanted cells (Jones et al., 2014a).

Model	Cells	Age	Route	Irradiated	Engraftment	Differentiate	Bones	Reference
COL1A1 mini-gene	WT mouse bone marrow MSC	3 weeks	i.p.	Yes	Yes (4-19%)	Not measured	Not assessed	Pereira, 1998
Oim/oim	GFP+ WT mouse osteo-progenitors	neonatal	i.v.	Yes	Yes	Yes	Not assessed	Wang, 2006
Oim/oim	GFP+ WT mouse osteo-progenitors	neonatal	i.v.	Yes	Yes	Yes	Not assessed	Li, 2007
Oim/oim	Luciferase+ human fetal blood MSCs	fetal	i.p.	No	Yes (5%)	Yes	< fracture rate > bone quality	Guillot, 2008
Heterozygous Brtl IV	GFP+ WT mouse bone marrow MSC	fetal	i.h.	No	Yes	Yes	> bone quality	Panaroni, 2009
Oim/oim	GFP+ WT mouse bone marrow MSC	Neonatal	i.f. + collagen gel	Yes	Yes	Yes	New bone formation > bone quality	Li, 2010
Oim/oim	Human fetal blood MSCs	fetal	i.p.	No	Yes (5%)	Yes	< fracture rate > bone quality	Vanleene, 2011
Oim/oim	SDF-primed human fetal blood MSCs	neonatal	i.p.	No	Yes (5-10%)	Yes	< fracture rate > bone quality	Jones, 2012
Oim/oim	GFP+ WT mouse bone marrow MSC	2-3 months	i.f.	Yes	Yes	Yes	New bone formation	Pauley, 2014
Oim	Human fetal early chorionic MSCs	neonatal	i.p.	No	Yes	Yes	< fracture rate > bone quality	Jones, 2014

Table 3. Preclinical studies of cell therapy for OI.

Cell therapy studies in OI mouse models. i.p.: intraperitoneal, i.v.: intravenous, i.h.: intrahepatic, i.f.: intrafemur. Adapted from (Jones and Guillot, 2013).

1.6 Cell therapy for OI in clinics

Following the promising results of the preclinical studies for cell therapy in OI, which report engraftment and therapeutic benefits in mouse models of the disease, different attempts to translate this approach have been made. Since 1999, ten children with severe OI have been transplanted with MSCs, either before or after birth. The limited clinical experience reported so far and ongoing efforts are summarised here.

1.6.1 Postnatal transplantations

In 1999, three children with severe deforming type III OI were transplanted in the second or third year of life (13-32 months of age) with bone marrow MSCs derived from HLA-matching or single-antigen-mismatched siblings (5.7-6.2 $x10^8$ cells/kg), following ablative conditioning. Opportunistic bone biopsies were collected from two of the transplanted children and engraftment of donor cells was estimated around 2%. Despite the low level of engraftment, an increase in bone mineral content was observed in all three children, ranging from 45% to 77% gain of total body mineral content in the first 6 months after transplantation. The three patients showed a 2-2.5 cm growth in the first 3 months and two children grew in line with the normal median growth velocity for their age in the first 6 months. A dramatic decrease in fracture rate was also reported. In the year before transplantation, two children reported more than 20 fractures, which decreased to 2-3 in the first 6 months after the procedure and none were reported in the following 6 months. In terms of toxicity, one child experienced pulmonary insufficiency, sepsis and developed an hygroma, all of which were resolved (Horwitz et al., 1999a).

Two years later, the authors reported a 36-month follow-up of two patients and an additional child, which also included clinical data from two non-transplanted age-matched OI children as controls. The transplanted children showed a showed a median 7.5-cm growth in the first 6 months, compared to the 1.25 cm observed in control children in the same period of time. After this initial improvement, however, growth reached a plateau. The number of fractures decreased from a median of 10

before treatment to a median of 2 at 12 months after transplantation, while in control children it remained constant at 4. Six children, who had previously undergone bone marrow transplantation as part of previous studies, were enrolled in a study to assess the therapeutic effects of repeated MSC transplantations. Two infusions (1-5 x 10^6 cells/kg) with the same MSC donor used for the previous study were performed 18-34 months after the original treatment. Five children showed engraftment of donor cells in bones. In the six months after transplantation, growth rates showed a marked acceleration (60-94% of the predicted median). No adverse effects were observed apart from one child suffering from a skin rash (Horwitz et al., 2002).

1.6.2 Prenatal transplantations

OI can be diagnosed during mid-pregnancy ultrasound scan, allowing the possibility of stem cells transplantation before birth. Advantages of prenatal, rather than neonatal, transplantations are numerous, including the possibility to limit the damages of severe congenital disorders, the lack of requirement of ablative therapy, the more permissive fetal environment for donor cell homing and the stoichiometric advantage in terms of number of cells required, given by the small size of the fetus (Le Blanc et al., 2005).

Only two cases of *in utero* MSC transplantation for OI have been reported so far. The first fetus was diagnosed with severe type III OI and was transplanted at 32 weeks of gestation with allogeneic mismatched MSCs derived from the liver of a 10-week aborted fetus (6.5 x 10^6 cells), via the umbilical vein under ultrasound guidance. Since donor cells were of male origin and the recipient fetus was a female, engraftment analyses were performed by assessing the karyotype of cells in a bone biopsy. A median of 7.4% of Y chromosome-positive cells was estimated. No immune rejection of donor cells was detected and the child had only three fractures in the first 24 months of life (Le Blanc et al., 2005). The child, who also had pharmacological treatment with bisphosphonates, then grew according to the predicted growth rate until 8 years of age, despite having several complications and more long bone fractures. At 6 years of age, donor cells were no longer detected in an opportunistic bone biopsy and the child's growth slowed down. At 8 years of age, the child

underwent a second transplantation (2.8 x 10^6 cells/kg, intravenously injected) with the same fetal liver MSCs used in the intrauterine setting. In the two years after the postnatal transplantation no new fractures were observed and the child grew normally and had a good quality of life. Engraftment was very low after 9 months, with about 0.003% Y chromosome-positive cells detected in a bone biopsy. A child with the same mutation of the transplanted patient, who was treated with bisphosphonates and did not undergo MSCs transplantation, died at 5 months of age (Götherström et al., 2014).

The second child was diagnosed with OI after detection of intrauterine bone fractures during an ultrasound scan at mid pregnancy. The fetus was transplanted at 31 weeks with allogeneic fetal liver MSCs derived from a 7-week-old terminated fetus (30 x 10^6 cells/kg) via the hepatic vein. The baby was born without complications and grew without fractures until 1 year of age, when her growth arrested. A second MSC transplantation was performed at 19 months of age with the same cells used previously (10 x 10^6 cells/kg), which allowed the girl to grow just below the normal curve. No information on engraftment is available in this second case. A therapy with bisphosphonates was given in both cases (Götherström et al., 2014).

1.6.3 Phase I/II clinical trial

Stemming from the promising results obtained by the many preclinical studies and the limited clinical experience so far, the first *in utero* stem cell transplantation clinical trial will be initiated in 2017, led by the Karolinska Institute. The Boost Brittle Bones Before Birth (BOOSTB4) phase I/II trial is funded by the European Commission Horizon 2020 and the Swedish Research Council and will assess the safety and efficacy of prenatal and neonatal transplantation of MSCs for the treatment of severe OI (types III and IV). The study is a multicentre study and four clinical hubs are taking part: the Karolinska Institute in Sweden, UCL/Great Ormond Street Hospital in the UK, Leiden University in the Netherlands and Uniklinik Koln in Germany. The trial consortium (http://boostb4.eu) is going to compare the safety and benefits of *in utero* transplantation with postnatal transplantation at 4 months of age and absence of treatment in age and mutation matched controls (Chitty et al., 2016).

1.7 Thesis aims and research questions

The main aim of the work presented in this thesis is the investigation of the therapeutic potential of human amniotic fluid stem cells (AFSCs) transplantation in the *oim* mouse model of osteogenesis imperfecta (OI).

Moreover, this work aims at investigating the mechanisms of action of human AFSCs transplanted into the *oim* mouse model of OI, leading to therapeutic effects.

The last aim of this work is the establishment of a protocol for the isolation and differentiation of human fetal osteoblasts that could be used to model human bone *in vitro*.

Specifically, the following research questions have been addressed:

- Do human AFSCs transplanted into *oim* mice engraft and differentiate *in vivo?* (Chapter 3)
- Does transplantation of human AFSCs into *oim* mice ameliorate *oim* bone biomechanical properties and bone structure? (Chapter 4)
- Do human AFSCs transplanted into *oim* mice have a paracrine effect on *oim* osteoblasts? (Chapter 5)
- Can AFSC-conditioned medium recapitulate *in vitro* the effect of AFSCs seen *in vivo?* (Chapter 6)
- Is the human fetal calvaria a source of osteoblasts that can be used to model human bone formation *in vitro?* (Chapter 6)

Chapter 2

Materials and methods

2.1 Cell biology and animal work

2.1.1 Cell culture of human amniotic fluid and fetal liver MSC

Human first and second trimester AFSCs were isolated by a previous member of the lab (Dr. Dafni Moschidou), following ultrasound-guided amniocentesis of normal pregnancies, using the isolation protocol described previously (Moschidou et al., 2013b). Informed consent for the use of cells for research was obtained and ethical approval was given by the Research Ethics Committees of Hammersmith & Queen Charlotte's Hospitals (08/H0714/87), in compliance with national guidelines. Briefly, amniotic fluid was centrifuged and pelleted cells were plated in Dulbecco's Modified Eagle Medium – high glucose (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (Biosera), 2 mM L-glutamine (Gibco), 50 IU/ml penicillin and 50 mg/ml streptomycin (Gibco) (From now referred to as DMEM). Cells were incubated at 37°C in a humidified incubator with 5% CO_2. Following adhesion, cells were detached with TrypLE dissociation reagent (Gibco) and sorted for C-KIT expression. Selected cells were expanded and cryopreserved in liquid nitrogen.

First trimester human fetal liver MSCs were isolated from aborted tissues by Dr. Dafni Moschidou following the protocol described previously (Guillot et al., 2008). Briefly, fetal liver tissue was minced through a nylon filter and single cell suspension was plated in DMEM an incubated in a humidified incubator at 37°C and 5% CO_2. After 72 hours medium was changed to remove non-adherent cells and remaining cells were expanded and cryopreserved in liquid nitrogen until use. Cells for the experiments were expanded by seeding them at 10,000 cells/cm^2, changing the medium every 3 days. Cells were passaged when reaching 70% of confluence by TrypLE detachment. Experiments were performed using AFSCs and fetal liver MSCs at passage 5-8. When cryopreservation was needed, cells were detached with TrypLE, centrifuged and resuspended in freezing medium consisting of 50% fetal bovine serum (Biosera), 40% DMEM (Invitrogen) and 10% dimethyl sulfoxide (DMSO, Sigma). Cells were then transferred into cryo-vials and stored in liquid nitrogen.

2.1.2 Transplantations in the oim model

Animal work was carried out by Dr. Dafni Moschidou and Dr. Gemma Jones. Oim homozygous and wild-type colonies were established after sequencing the *oim* fragment in offspring of *oim* heterozygous male and female mice (B6C3Fe a/a-Col1a2^{oim}/ Col1a2^{oim}, Jackson Laboratory). Offspring were weaned at 30 ± 1 day. At 3-4 days of age, homozygous *oim* mice were transplanted intraperitoneally with human AFSCs (10^6 cells per mouse, in 20µl of cold PBS, passage 5-8, derived from one donor) and were culled at 8 weeks of age, together with age-matched non-transplanted *oim* and wild-type mice as controls.

2.1.3 Conditioned medium preparation

Conditioned medium was produced by culturing AFSCs (20,000 cells/cm^2) for 48 hours in expansion medium (expansion medium only or supplemented with 5 ng/ml TGF-β or TNFα for 24 hours), collecting the culture medium and concentrating it using Amicon Ultra filters, pore size 3kDa (Merck) to reach a concentration of 200 times. Briefly, 4 ml of culture medium was collected from the AFSC culture wells, transferred into the Amicon filters and centrifuged for 40 minutes at 4,000 x g using a swinging bucket rotor (Thermo Scientific). The resulting concentrated medium was collected from the filter and dissolved in 4ml of fresh culture medium to produce conditioned medium.

2.1.4 MG-63 osteosarcoma cell line culture

MG-63 osteosarcoma cells were obtained from the American Type Culture Collection (ATCC). Cells were seeded at 20,000 cells/cm^2 in DMEM in a humidified incubator at 37°C and 5% CO_2 and passaged at 80% confluence by TrypLE incubation. Expansion medium was changed every 3 days.

2.1.5 Mouse osteoblast isolation and culture

Osteoblasts were isolated from calvarial bone of 3-day old *oim/oim* mice, using a previously established protocol (Taylor et al., 2014), consisting of sequential

enzymatic digestions (**Figure 7**). Briefly, after the skin, brain tissue and cartilage were carefully removed with a scalpel, the calvaria was cut in half, washed with PBS (Biosera) and incubated for 10 minutes in 0.25% trypsin-EDTA solution (Gibco) at 37°C, in order to lyse the proteins of the outer layer. The resulting digestion was then discarded and the calvarial pieces were washed with FBS-containing medium in order to inactivate trypsin and incubated for 30 minutes in a 0.2% collagenase type II solution in Hank's balanced salt solution (HBSS, Gibco) at 37°C, to loosen the collagen structure and release cells. The second digestion was also discarded and bone pieces were incubated for an additional 60 minutes in 0.2% collagenase type II solution in HBSS, to release the osteoblasts. The last digest was plated into a 75 cm^2 flask in Alpha-Modified Essential Medium (αMEM, Gibco) supplemented with 10% FBS (Biosera), 2mM L-glutamine (Gibco) and 50 IU/ml penicillin and 50 mg/ml streptomycin (Gibco) (from now described as αMEM). Cells were allowed to grow until confluent (6 days) in a humidified incubator at 37°C and 5% CO_2, replacing medium every 3 days. Cells were cultured by seeding them at 10,000 cells/cm^2 and passaged by trypsin-EDTA detachment. Experiments were performed with cells at passages 2-3. When cryopreservation was needed, cells were detached with trypsin-EDTA, centrifuged and resuspended in freezing medium consisting of 50% fetal bovine serum (Biosera), 40% αMEM (Gibco) and 10% dimethyl sulfoxide (DMSO, Sigma). Cells were then transferred into cryo-vials and stored in liquid nitrogen.

2.1.6 Human fetal osteoblasts isolation and culture

Human fetal osteoblasts were collected by fetal calvarial tissue, obtained by terminated pregnancies, collected by the MRC/Wellcome-Trust funded Human Developmental Biology Resource (HDBR) at the UCL GOS Institute of Child Health. Samples had gestational ages ranging between 9 and 21 weeks post conception and had all a normal karyotype. Upon collection, calvarial tissue was dissected and soft tissues discarded. In order to achieve effective isolation of human fetal osteoblasts, the protocol used for mouse osteoblast isolation (Taylor et al., 2014) was optimised by testing different cycles of trypsin and collagenase digestion (See **Figure 7** for comparison with the original mouse protocol). Bone tissue was dissected into small

fragments of about 0.5 cm^2 and washed thoroughly with PBS. Bone fragments were subsequently incubated with 0.25% trypsin-EDTA solution to digest the outmost layer of proteins. Different 15-minute cycles of 0.25% trypsin-EDTA solution were tested (See Results Chapter 6), followed by different combinations of 30-minute cycles of 0.2% collagenase type II in HBSS incubation in a humidified incubator at 37°C and 5% CO_2. The resulting final digestions was plated in αMEM and cultured changing medium every 3 days until confluency. Isolated human fetal osteoblasts were cultured by seeding them at 10,000 cells/cm^2 in αMEM and passaged upon confluency. Experiments were performed with cells of passage 2-5. A biobank of 24 human fetal osteoblast samples derived from calvarial bones was built, by freezing cells at early passages (1-3). Cells were detached by TrypLE (Gibco) and transferred to liquid nitrogen in freezing medium consisting of 50% fetal bovine serum (Biosera), 40% αMEM (Gibco) and 10% dimethyl sulfoxide (DMSO, Sigma).

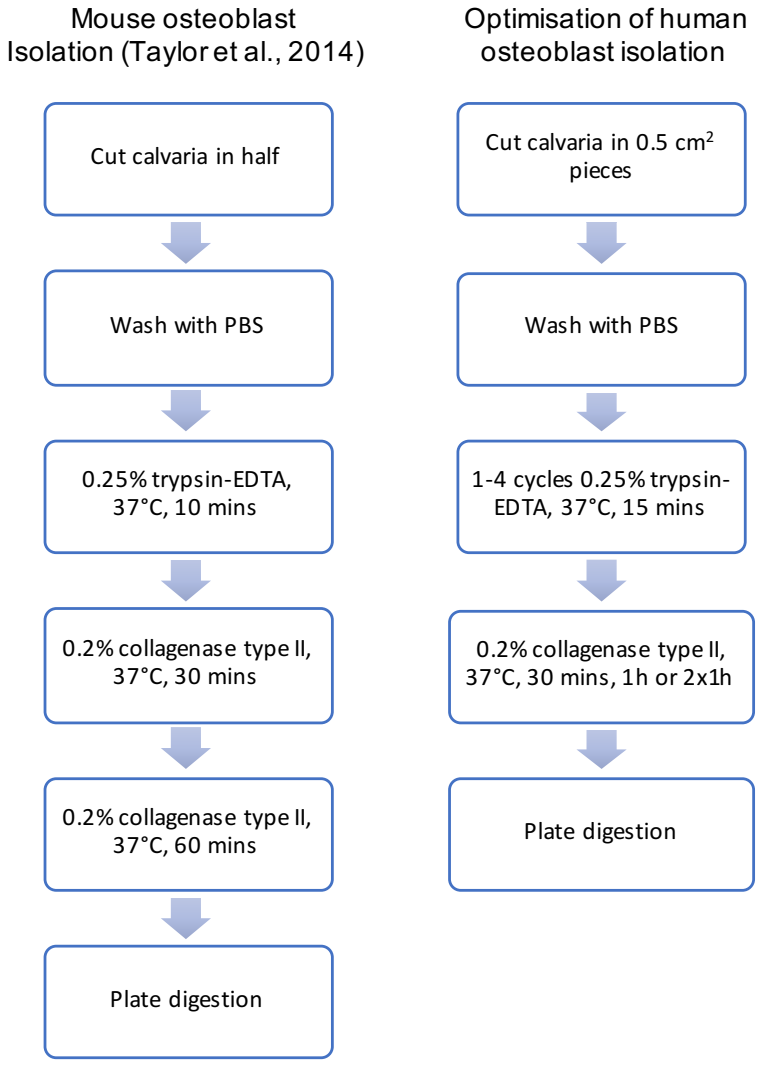


Figure 7. Mouse and human osteoblast isolation protocols.

Diagram comparing the protocols used for mouse and human osteoblast isolation.

2.1.7 Osteogenic differentiation

Human and mouse cells were seeded at 20,000 cells/cm^2 in 6 or 12-well plates in expansion medium (DMEM or αMEM). Upon confluency, cells were cultured in osteogenic medium for up to 3 weeks, changing medium every 3 days. Osteogenic medium contained 0.2 mM ascorbic acid (Sigma), a cofactor for the biosynthesis of collagen, 10 nM dexamethasone (Sigma), which enhances osteogenic differentiation, and 2.5-10 mM β-glycerophosphate (Sigma), which acts as a substrate to alkaline phosphatase to provide the orthophosphate needed for mineralisation (refer to individual chapters for β-glycerophosphate concentrations used with the different cell types). Previous studies showed that finding the correct β-glycerophosphate concentration is crucial to obtain *in vitro* bone formation and to avoid non-specific mineral deposition (Orriss et al., 2012; Taylor et al., 2014). Terminated cultures were fixed with 70% ethanol for 5 minutes and then let to air-dry for 30 minutes.

2.1.8 Alizarin red staining

Mineralised cultures were stained with 1% w/v alizarin red in PBS (Sigma) for 5 minutes, washed three times with 50% ethanol to remove excessive staining and let to air-dry for 30 minutes. Cultures were then imaged by a high-resolution flat-bed scanner (Epson).

2.1.9 Adipogenic differentiation

Cells were seeded at 10,000 cells/cm^2 and cultured in DMEM until 90% confluent. Expansion medium was then replaced by adipogenic permissive medium (StemPro adipogenesis differentiation medium, Gibco), which was changed twice a week for 3 weeks. Cultures were then fixed with 4% paraformaldehyde for 15 minutes and stained with Oil Red O.

2.1.10 Oil Red O staining

Oil Red O (Sigma-Aldrich) was prepared at 0.3% concentration in isopropanol (VWR) and subsequently diluted in distilled water in a ratio of 3:2 and filtered. Fixed cells

were washed with 60% isopropanol and then incubated for 15 minutes with the Oil Red O solution at room temperature. Finally, cells were washed thoroughly with distilled water to remove excessive stain and cultures were imaged with an inverted phase contrast microscope (Olympus IMT-2) and a colour digital camera (Zeiss AxioCam).

2.1.11 Growth rate

Growth rate of first trimester AFSCs, fetal liver MSCs and human fetal osteoblasts of different gestational ages was measured by estimating their cumulative population doublings, following the protocol described in (Guillot et al., 2007). Briefly, cells were plated at low density (5,000 cells / cm^2) in 12 well plates in triplicate. Every 3 days cells were detached, counted in a haemocytometer and subsequently seeded at the same density. This was repeated for 3 weeks and the number of population cell doublings was measured by counting the number of adherent cells before and after each passage. Doubling time was measured with the formula $DT=t/(Log_2[y/m]$ with t being time in culture, y the number of cells at the end of culture and m the number of cells at the beginning of culture for each passage.

2.2 Molecular Biology

2.2.1 Flow cytometry

Surface marker expression was assessed by flow cytometry. AFSCs were detached, using TrypLE, blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS and incubated with specific conjugated primary antibodies (See **Table 5**) for 1 hour at 4°C. Cells were then washed in 1% BSA in PBS and analysed by FACScalibur flow cytometer (Becton Dickinson). Immunoglobulin G (IgG) primary antibody-specific isotypes were used as negative controls. Data were analysed with Flowjo software (Tree Star).

2.2.2 RNA isolation

Total RNA was isolated from cells using the RNeasy mini kit (Qiagen) following manufacturer's instructions and eluted in RNase-free water (Qiagen). RNA from mouse *oim* and wild-type bones and soft organs was extracted by grinding tissues using a rotor stator homogeniser with the addition of Trizol (Invitrogen), followed by chloroform (Sigma-Aldrich). The aqueous phase was then mixed with isopropanol to precipitate RNA and centrifuged. 70% ethanol was then used to wash the pellet, which was then resuspended in RNase-free water. In both protocols, RNA was then quantified by measuring absorbance at a wavelength of 260 nm using a NanoDrop spectrophotometer (Thermo Scientific).

2.2.3 cDNA synthesis

Complementary DNA (cDNA) was produced incubating RNA with random primers (Promega) at 70°C for 5 minutes and subsequently adding Moloney-Murine Leukemia Virus reverse transcriptase (M-MLV RT, Promega) and dNTPs (Promega), in presence of RNase inhibitor (Promega). The reaction was incubated at 37°C for 1 hour and then frozen to stop the reaction. cDNA synthesis for gene arrays was performed using a RT^2 First Strand Kit (Qiagen), according to manufacturer's instructions.

2.2.4 Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qPCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems) and specific pairs of forward and reverse primers (See **Table 4** for sequences). Amplification of cDNA was obtained using a StepOne Plus thermocycler (Applied Biosystems) with the following cycling conditions: 1 x 2 mins 94°C, 40 x (15 sec 94°C and 1 min 60°C). GAPDH or β-actin were used as internal controls to normalise the expression of the target genes in each sample. Unless specified, data were collected in triplicate and were analysed according to the $2^{-\Delta\Delta Ct}$ method. Refer to individual figures for sample number. To measure AFSC engraftment level on *oim* bones and organs, the ratio of the expression of human β-actin to the expression of total human + mouse β-actin was calculated

and used to estimate the level of human:mouse chimerism, as described in (Guillot et al., 2008; Jones et al., 2014). Areas of β-actin gene sequence (accession number: NM_001101) that are present in the human gene but not in the mouse gene were used to design the human-specific primers and specificity was verified by the lack of amplification in non-transplated *oim* cDNA (Guillot et al., 2008; Jones et al., 2014). Samples were considered positive with a human specific β-actin Ct value below 36 at a threshold of 0.13ΔRn.

2.2.5 Gene array

The expression of multiple genes in transplanted and non-transplanted *oim* bones was assessed by gene arrays, which allow the analysis of expression of a panels of mouse genes simultaneously. The arrays used were commercially available mouse-specific osteogenesis and osteoporosis RT^2 Profiler mouse PCR arrays (Qiagen). Two internal controls (housekeeping genes β-actin and Hsp90ab1) were used to normalise the level of expression to total cDNA amount in each sample. The arrays were run using a HT7900 Fast Real-Time PCR cycler (Applied Biosystems) at the UCL GOS Institute of Child Health Genomics facility. Cycling conditions were the following: 1 x 10 mins 95°C, 40 x (15 sec 95°C and 1 min 60°C). Data were analysed using the GeneGlobe Data Analysis Centre web tool included in the arrays (Qiagen) and expression of genes following AFSC transplantation was plotted as a fold change from the level of expression in non-transplanted animals (2-fold change was selected as a threshold cut-off).

2.2.6 Protein extraction and quantification

Mouse femurs were ground to powder from frozen with a rotor-stator homogeniser and total protein was extracted using RIPA buffer, containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and 0.004% sodium azide, supplemented with protease inhibitor cocktail (all Sigma). The concentration of proteins was measured using the BCA protein assay (Thermo Scientific), and reading absorbance at a wavelength of 560 nm, using BSA to build a standard curve.

2.2.7 Western blot

Protein lysates were separated on an 8-10% SDS-PAGE gel and blotted onto a nitrocellulose transfer membrane (GE Healthcare) for an hour at 4°C. The membrane was then blocked for one hour with 5% milk PBS-T (0.1% Tween20 in PBS) and incubated overnight at 4°C with primary antibodies raised against the proteins of interest (See **Table 5** for specific antibodies). Incubation with secondary antibodies conjugated to horseradish peroxidase (HRP) was performed at room temperature for one hour (See **Table 6** for specific antibodies). Detection was achieved by incubation with the Enhanced Chemiluminescent developing solution (ECL substrate, Thermo Scientific). Bands were imaged with a Chemidoc detection system (Bio Rad) and quantified using ImageJ software, based on band densities. β-actin was used as a loading control (Santa Cruz).

2.3 Histology and microscopy

2.3.1 Immunohistochemistry

Mice tibiae were dissected, decalcified for 2 weeks in 10% EDTA at pH 7.4 and embedded in paraffin. 4μm thick sections were cut, deparaffinised in xylene, and rehydrated. Heat induced antigen retrieval was performed on a hot plate with citrate buffer at pH 6 followed by serum-free blocking agent. Slides were subsequently incubated with Peroxidase Blocking Reagent (Dako) and blocked with 5% goat serum in PBS. Staining was performed with human-specific primary antibody (See **Table 5**) at 4°C overnight. Slides were then incubated with HRP-conjugated secondary antibody followed by DAB+ substrate-chromogen staining (Dako) and imaged by a Zeiss Axioplan2 microscope with Zeiss Axiocam HRc colour camera (UCL GOS Institute of Child Health microscopy facility; collaboration with Ms Mahrokh Nohadani).

2.3.2 Haematoxylin and eosin staining

Mice tibiae were dissected, decalcified for 2 weeks in 10% EDTA at pH 7.4 and embedded in paraffin. 4µm thick sections were cut, deparaffinised in xylene, and rehydrated. Sections were then stained with haematoxylin (Harris modified, Thermo Scientific) for 5 minutes. Then they were rinsed in 1% acid alcohol (37% HCl into 70% ethanol) and counterstained in 1% eosin Y in distilled water (Sigma) for 3 minutes. Dehydration was then performed with increasing concentrations of alcohols and sections were incubated in xylene for 6 minutes before mounting with DPX (Sigma).

2.3.3 Immunofluorescence

Mice tibiae were dissected, decalcified for 2 weeks in 10% EDTA at pH 7.4 and embedded in paraffin. 4µm thick sections were cut, deparaffinised in xylene, and rehydrated. Heat induced antigen retrieval was performed on a hot plate with citrate buffer at pH 6 followed by serum-free blocking agent. Slides were subsequently blocked with 5% goat serum in PBS and incubated with a human-specific primary antibody (See **Table 5**) overnight at 4°C, followed by incubation with FITC-conjugated secondary antibody (See **Table 6**). Slides were then mounted with VectaShield containing DAPI (Vector) to visualise nuclei and imaged by a Leica DM LM fluorescence microscope with CoolSnap monochrome camera (UCL GOS Institute of Child Health microscopy facility; collaboration with Ms Mahrokh Nohadani).

2.3.4 Two-photon lifetime imaging

An upright LSM 510 microscope (Carl Zeiss) with a water-dipping objective (1.0 NA × 40) was used for the two-photon lifetime imaging of collagen produced by human fetal osteoblasts, in collaboration with Dr. Thomas Blacker and Prof. Michael Duchen, UCL Department of Cell and Developmental Biology. A 650-nm short-pass dichroic and a 460±25 nm emission filters were used. A Chameleon (Coherent) Ti:sapphire laser provided two-photon excitation (720 nm for collagen autofluorescence and 920 nm for second harmonic generation). Fluorescence lifetime measurements were recorded with a SPC830 FLIM module (Becker and Hickl).

2.4 Micro-computed tomography

2.4.1 Scan acquisition

Tibiae were dissected and fixed in 10% neutral buffered formalin for 24 hours, subsequently washed with PBS and preserved in 70% ethanol until scanned. Bones were scanned with a Bruker Skyscan 1172 micro-CT scanner (by Dr. Michelangelo Corcelli, Bone Biology lab, UCL Department of Cell and Developmental Biology), in small plastic tubes containing 70% ethanol. Scans were performed using a beam energy of 49 KV and a flux 200 µA, a 0.5 mm aluminium filter and an isotropic pixel size of 5.06 µm. Each image was acquired with an exposure time of 590 ms, frame averaging of 3 and a 0.4-degree rotation step with a total rotation of 180 degrees and using medium camera resolution (2000x1048px). Random movement was set to ON and flat field correction was applied.

2.4.2 Reconstruction

The Skyscan NRecon software (Bruker) was used to reconstruct the scans. The following reconstruction settings were applied: ring artifacts reduction was enabled and set to 1, smoothing was enabled and set to 1 to reduce background noise and beam hardening correction was enabled and set to 25%.

2.4.3 Analysis

Skyscan CT Analyser (Bruker) was used to measure the morphometric parameters. **Figure 8** shows the area of the tibia analysed for the different trabecular and cortical parameters. Trabecular bone was analysed taking in consideration a 1 mm thick region located 0.25 mm below the end of the tibial growth plate. Cortical thickness was measured at 1.75 mm, 2.25 mm and 2.75 mm below the end of the growth plate, whereas cortical porosity was assessed in 0.5 mm thick region at 2.25 mm below the end of the growth plate. Subchondral parameters were analysed in an area of interest of 0.5 mm from the beginning of the growth plate and the thickness of primary ossification was calculated manually on Data Viewer as an average of three

measurements. The parameters assessed were: percentage bone volume (BV/TV/%), trabecular thickness (Tb.Th/mm), trabecular number (Tb.N/mm-1), trabecular separation (Tb.Sp/mm), trabecular pattern factor (Tb.Pf/mm-1), cortical thickness (Ct.Th/mm) and cortical porosity (Ct.Po/%).

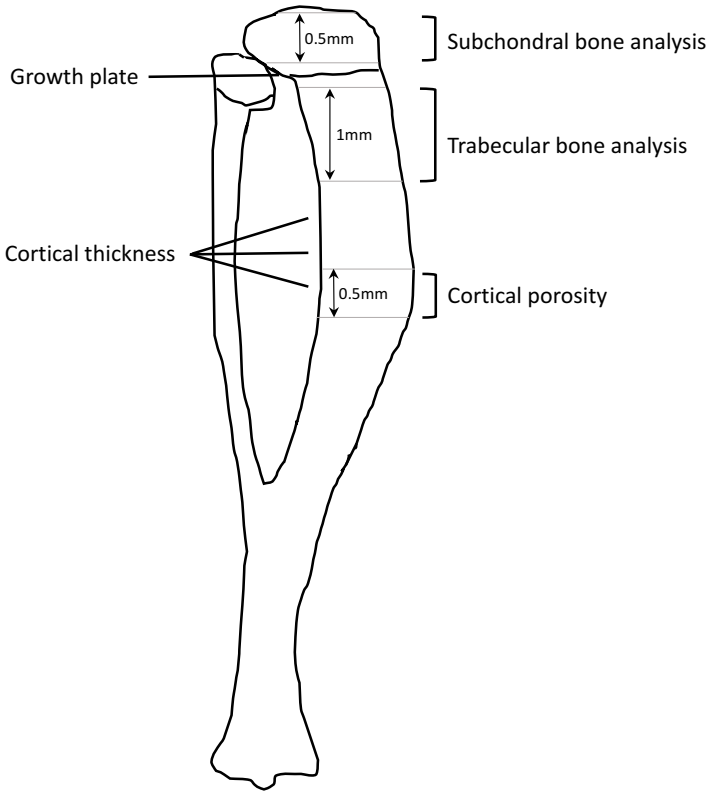


Figure 8. Diagram showing the sites of micro-CT parameter measurements in mouse tibia.

Micro-CT morphological parameters, including percentage bone volume (BV/TV/%), trabecular thickness (Tb.Th/mm), trabecular number (Tb.N/mm^{-1}), trabecular separation (Tb.Sp/mm) and trabecular pattern factor (Tb.Pf/mm^{-1}), were measured for subchondral bone in a region of interest (ROI) of 0.5 mm located above the growth plate of mouse tibia. The same parameters were measured for trabecular bone in a ROI of 1mm located 0.25mm below the growth plate. Cortical thickness (Ct.Th/mm) was assessed in three different locations, 1.75, 2.25 and 2.75mm below the growth plate. Cortical porosity (Ct.Po/%) was measured in a ROI of 0.5mm located 2.25 mm below the growth plate.

2.4.4 BMD and TMD analysis

Bone mineral density and tissue mineral density measurements were performed by calibrating the SkyScan CT Analyser software using standards of known calcium hydroxyapatite content as a reference (initial calibration performed by Mr Nick Corps, Bruker UK). Two phantoms of 0.25 and 0.75 g/cm^3 of calcium hydroxyapatite (Bruker) were scanned with the same settings used for the samples and the calibration was performed by the Hounsfield unit system. Bone mineral density was measured in the trabecular ROI, while tissue mineral density in the cortical ROI (**Figure 8**).

2.5 Statistics

Data were expressed as mean ± SEM (standard error of the mean). Data were analysed by unpaired two-tailed Student's t-test or one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* correction using GraphPad (Version 6, GraphPad Sotware Inc.). $P< 0.05$ was considered significant. Chi-squared with Yates correction and to one degree of freedom was used to compare fracture incidence.

2.6 Primers

Gene	Forward	Reverse	Product number
Mouse osteogenesis gene array	-	-	Qiagen RT^2 Profiler PAHS-026Z
β-actin (human)	5'-CTGGAACGGTGAA GGTGACA-3'	5'-AAGGGACTTCCTGT AACAATGCA-3'	
β-actin (human/mouse)	5'-GCTCCTCCTGAGCG CAAGTA-3'	5'-GATGGAGGGGCCG GACT-3'	
β-actin (mouse)	-	-	Qiagen Quantitect QT00095242
Alp1	-	-	Qiagen Quantitect QT00157717
Cdkn1a	-	-	Qiagen Quantitect QT00137053
GAPDH	5'-AACAGCGACACC CACTCCTC-3'	5'-CATACCAGGAAAT GAGCTTGACAA-3'	
Osteocalcin (human)	5'-CCTCACACTCCTC GCCCTATT-3'	5'-CCCTCCTGCTTGG ACACAAA-3'	
Osteocalcin (mouse)	-	-	Qiagen Quantitect QT00259406
Osteopontin	5'-GCCGACCAAGGA AAACTCACTA-3'	5'-CAGAACTTCCAGA ATCAGCCTGTT-3'	
Osteprotegerin	-	-	Qiagen Quantitect QT00106757
RANKL	-	-	Qiagen Quantitect QT00147385
Runx2	-	-	Qiagen Quantitect QT00102193
Serpine1	-	-	Qiagen Quantitect QT00154756
TNFα	-	-	Qiagen Quantitect QT00104006

Table 4. List of primers.

List of primer sequences or product number in case of commercially available products. Custom made primers were designed by Dr. Gemma Jones.

2.7 Antibodies

Antibody	Host	Application	Dilution	Product Number
CD34-FITC	Mouse	Flow cytometry	1:10	BD Bioscience 555-821
CD45-FITC	Mouse	Flow cytometry	1:10	BD Bioscience 555-482
CD73-PE	Mouse	Flow cytometry	1:10	Miltenyi 130-095-182
CD90-APC	Mouse	Flow cytometry	1:10	Miltenyi 130-095-402
CD105-FITC	Mouse	Flow cytometry	1:10	Miltenyi 130-098-774
OSTEOPONTIN	Mouse	Immuno-fluorescence	1:100	Vector VP-O852
OSTEOPONTIN	Mouse	Immuno-histochemistry	1:50	Vector VP-O852
COLLAGEN I α2	Goat	Western blot	1:200	Santa Cruz sc8786
OSTEOCALCIN	Rat	Western blot	1:500	Takara M188
β-ACTIN	Mouse	Western blot	1:5000	Abcam ab3280

Table 5. List of primary antibodies.

List of primary antibodies used, the host species developed in, dilution used, application and product number.

Antibody	Host	Application	Dilution	Product Number
Anti-mouse HRP	Horse	Western blot	1:1000	Cell Signaling 7076
Anti-goat HRP	Donkey	Western blot	1:1000	Santa Cruz sc2020
Anti-rat HRP	Rabbit	Western blot	1:1000	Abcam ab6734
Anti-mouse FITC	Goat	Immuno-fluorescence	1:1000	Santa Cruz sc2082

Table 6. List of secondary antibodies.

List of secondary antibodies used, the host species developed in, dilution used, application and product number.

Chapter 3

Human AFSCs: characterisation and engraftment in the *oim* model

3.1 Introduction and objectives

Cell therapy is a promising option for the treatment of OI. A number of preclinical studies have shown the feasibility and efficacy of MSC transplantation in animal models of OI, both prenatally and postnatally (Pereira et al., 1998; Guillot et al., 2008; Vanleene et al., 2011; Jones et al., 2014a). Moreover, a small number of children with severe OI have been transplanted with adult bone marrow MSCs and fetal liver MSCs with encouraging results and without major adverse effects or immune rejection of the cells (Horwitz et al., 2001; Götherström et al., 2014).

One of the main hurdles for the further development of cell therapies for OI is the identification of a suitable source of MSCs for transplantation. The optimal cell source needs to be easily and ethically accessible. Moreover, since large numbers are needed for transplantation, the cells need to have a high proliferative capacity. Fetal MSCs have been demonstrated to have faster growth kinetics, reach senescence later and have a better differentiation capacity than adult MSCs (Guillot et al., 2008). However, cells from fetal tissues are isolated from terminated pregnancies and their use is limited due to ethical restrictions. In the past decade, AFSCs have been investigated as a potential and ethically available cell source for regenerative medicine purposes (De Coppi et al., 2007; Ramachandra et al., 2014).

The aim of this chapter is to characterise second trimester AFSCs and to assess engraftment levels and *in vivo* differentiation, when transplanted into *oim* neonatal mice.

More specifically, the first objective of this chapter is the characterisation of second trimester AFSCs, according to the MSC-defining criteria set by the International Society for Cellular Therapy (Dominici et al., 2006). The second objective is to compare the osteogenic potential of AFSCs to the one of fetal liver MSCs, which are considered the 'gold standard' as a cell source for the treatment of OI. The last objective is to measure the engraftment levels and *in vivo* differentiation of AFSCs transplanted into *oim* mice.

3.2 Results

3.2.1 Characterisation and *in vitro* osteogenic potential of human AFSCs

Human second trimester AFSCs (n=3) showed plastic adherence and grew in a monolayer. Cells exhibited the characteristic fibroblast-like spindle-shaped morphology that is typical of MSCs (**Figure 9**).

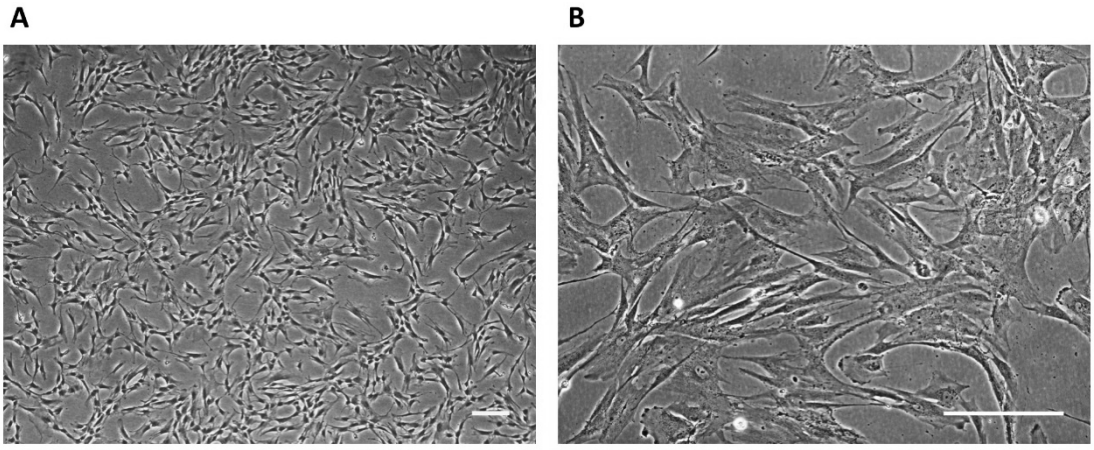


Figure 9. Morphology of human AFSCs.

A) Low and B) high magnification phase contrast images showing cell morphology of proliferating, undifferentiated AFSCs at passage 8. Scale bar: 200 µm.

Similar to other human fetal MSCs, human AFSCs are fetal cells that meet the minimum MSC-defining criteria defined by the International Society for Cellular Therapy (Dominici et al., 2006). Human AFSCs show above 95% co-expression of the MSC markers CD73 (ecto-5'-nucleotidase), CD90 (Thy-1) and CD105 (endoglin) (**Figure 10A**) and lack expression of the hematopoietic progenitor/endothelial marker CD34 (hematopoietic progenitor cell antigen CD34) and of hematopoietic marker CD45 (protein tyrosine phosphatase, receptor type C) (**Figure 10B**).

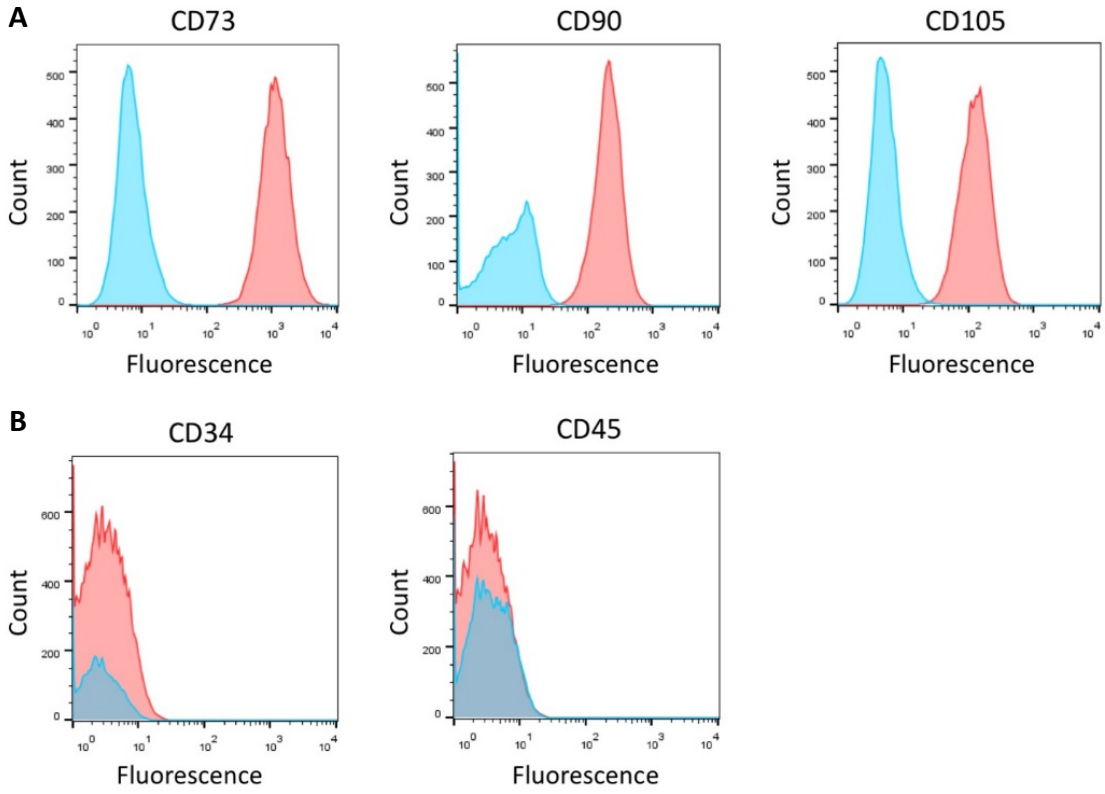


Figure 10. Human AFSCs express MSC markers.

Flow cytometry histograms showing AFSC (n=3, representative results shown) expression of MSC markers CD73, CD90 and CD105 (A) and lack of expression of endothelial markers CD34 and hematopoietic marker CD45 (B). Isotype control in blue.

MSCs are multipotent stem cells that are able to undergo adipogenic, chondrogenic and osteogenic differentiation (Dominici et al., 2006). Human AFSCs (n=3) cultured in adipogenic medium for 2 weeks differentiated into lipid-producing adipocytes. Lipid droplets were visualised by Oil Red O staining (**Figure 11**).

Human AFSCs (n=4) were able to differentiate into osteoblasts *in vitro*. A trabecular network of bone formed by human AFSCs was visible after 3 weeks of culture in osteogenic medium (**Figure 12A** and **C**). Alizarin red staining confirmed calcium deposition (**Figure 12B**).

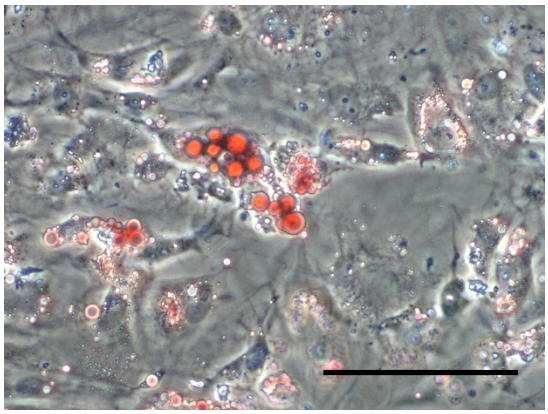


Figure 11. In vitro adipogenic differentiation of human AFSCs.

Oil Red O staining showing lipid droplets produced by human AFSCs cultured in adipogenic medium for 2 weeks. Scale bar: 100µm.

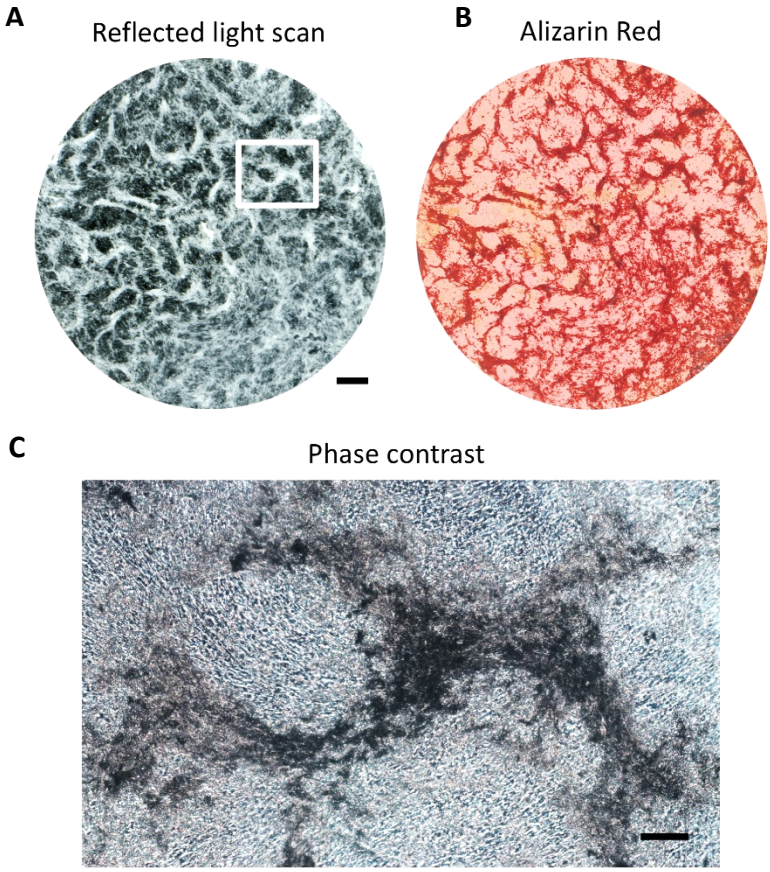


Figure 12. In vitro osteogenic differentiation of human AFSCs.

A) Unstained trabecular network of human AFSCs cultured in static 2D conditions in osteogenic medium for 3 weeks and visualised by scanning. B) Alizarin Red staining showing calcium deposits. Scale bar: 2mm. C) Trabecular network visualised by phase contrast microscopy. Scale bar: 300µm

3.2.2 Comparison of osteogenic potential of human AFSCs and fetal liver MSCs

Human AFSCs and human fetal liver MSCs derived from first trimester pregnancies (n=1) were cultured in monolayers in DMEM supplemented with 10% FBS. Human fetal liver MSCs were previously characterised by (Guillot et al., 2008). Both cell populations were plastic-adherent, showed similar fibroblast-like morphology and grew in a monolayer (**Figure 13**).

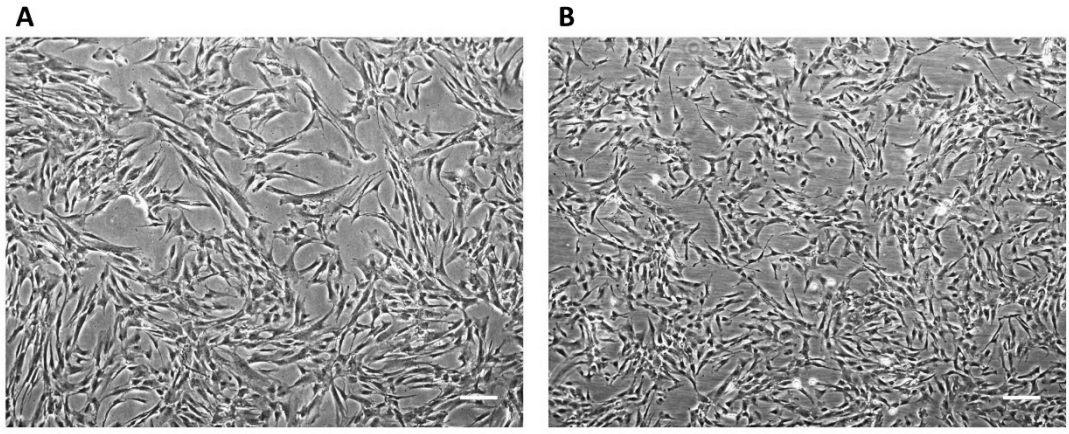


Figure 13. Morphology of human AFSCs and fetal liver MSCs.

Phase contrast image showing cell morphology of first trimester human AFSCs (A) and human fetal liver MSCs (B). Scale bar: 200µm.

The growth rate of AFSCs and fetal liver MSCs was assessed by seeding cells at low density (5,000 cells/cm^2) and charting their growth over a period of 18 days, throughout which the cell number was counted and the same number of cells re-seeded every 3 days. AFSCs grew approximately twice as fast as fetal liver MSCs. The population doubling time was 63.0 ± 4.5 hours for AFSCs and 127.6 ± 12.8 hours for fetal liver MSCs, P<0.01 (**Figure 14A**). Cumulative population doublings over the 18 days were 7.2 ± 0.2 for hAFSCs and 3.8 ± 0.3 for fetal liver MSCs, P<0.001 (**Figure 14B**).

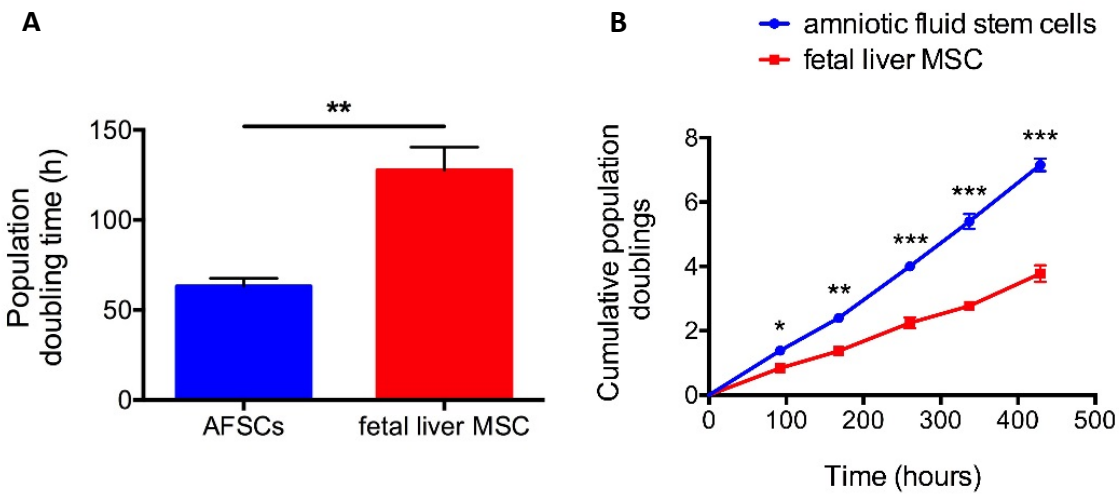


Figure 14. Human AFSCs grow faster than human fetal liver MSCs.

*A) Population doubling time of human AFSCs and fetal liver MSCs. B) Cumulative population doublings of human AFSCs and fetal liver MSCs measured for a period of 18 days. (n=1 run in triplicate) Data represent mean ±SEM * P<0.05, ** P<0.01 and *** P<0.001 (Student's t-test)*

The *in vitro* osteogenic potential of human AFSCs and fetal liver MSCs was assessed by culturing cells for 20 days in osteogenic permissive medium containing ascorbic acid, β-glycerophosphate and dexamethasone. Human AFSCs showed extensive bone formation characterised by mineralised trabecular structures, whilst human fetal liver MSCs did not show osteogenic differentiation (**Figure 15A**).

Alizarin red staining confirmed calcium deposition in human AFSCs and lack of mineral deposition in human fetal liver MSCs (**Figure 15B**).

The *in vitro* osteogenic differentiation potential of human AFSCs and fetal liver MSCs was also investigated by quantitative real-time PCR for expression of the osteogenic markers osteopontin and osteocalcin, after 10 days of differentiation. There was no significant difference in the expression of the two osteogenic genes, although osteocalcin, a marker of late osteogenic differentiation, was expressed at slightly higher levels in AFSCs than in fetal liver MSCs (**Figure 15C**).

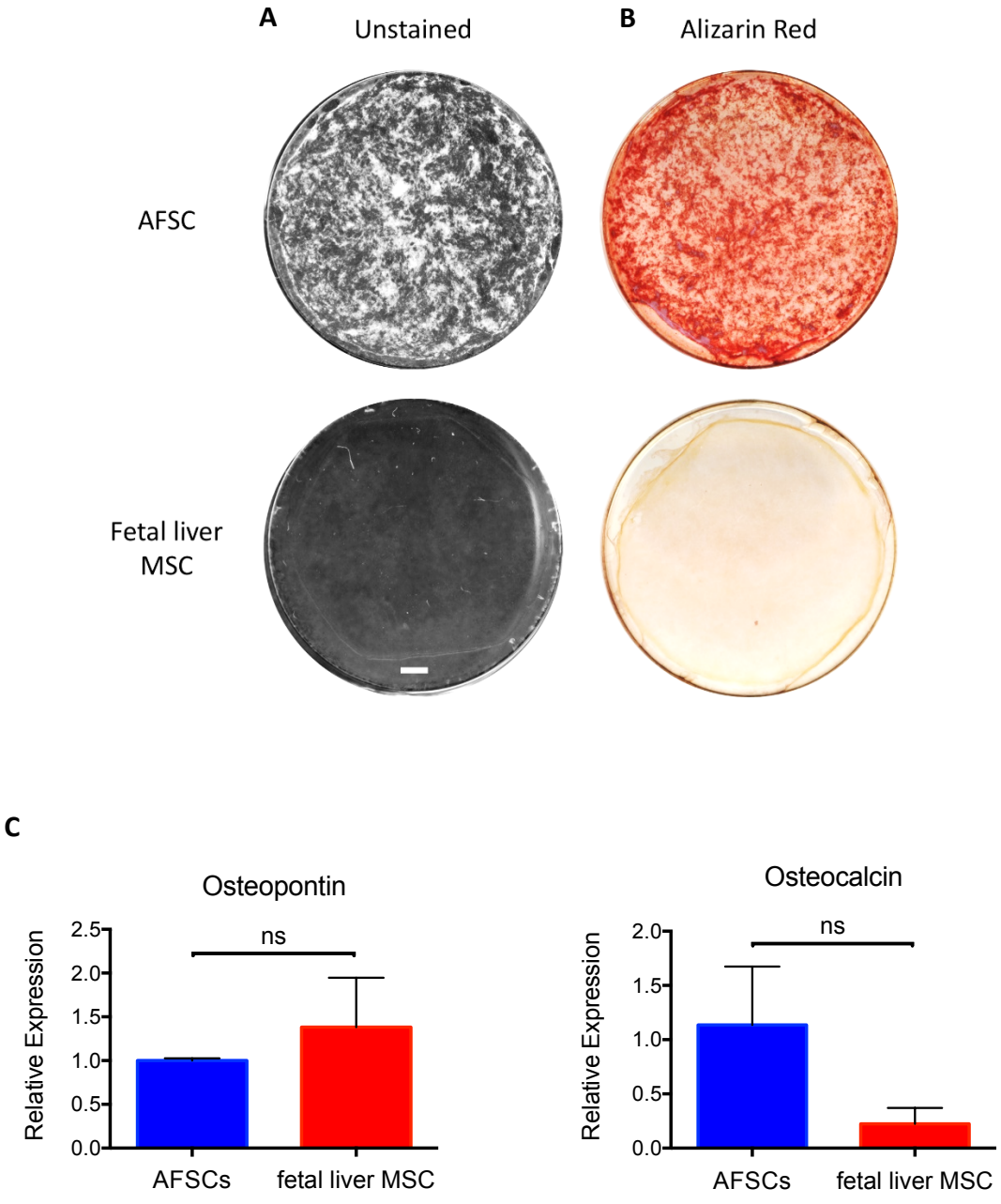


Figure 15. Human AFSCs have greater in vitro osteogenic potential than fetal liver MSCs.

A) Unstained osteogenic cultures of human AFSCs and fetal liver MSCs at day 20 in osteogenic medium. B) Alizarin Red staining showing calcium deposition. Scale bar: 2 mm. C) Quantitative real time PCR of expression of osteogenic genes after 10 days of differentiation. (n=1 run in triplicate) Data represent mean ±SEM. Student's t test.

3.2.3 Human AFSCs engraft into the bones of *oim* mice and differentiate into osteoblasts *in vivo*

Human AFSCs were transplanted intraperitoneally into neonatal *oim* mice (10^6 cells per mouse, transplantation and dissection performed by Dr. Jemma Jones). After 8 weeks, bones were harvested and engraftment was assessed by quantitative real time PCR. The level of engraftment was measured by the ratio of the expression of human β-actin over the total amount of human and mouse β-actin, as previously described (Guillot et al., 2008a; Jones et al., 2014) (See Materials and methods for details). Human donor cells were detected in bones of all of the 20 transplanted mice analysed. Transplanted human AFSCs preferentially engrafted into the epiphyses of the long bones, which is the site of active bone formation. To a lesser extent, donor cells also engrafted into the diaphysis and the bone marrow. Engraftment levels in the bone epiphysis were 1.8-fold higher than in the diaphysis (350.0 x 10^{-5} ± 17.9 vs 195.2 x 10^{-5} ± 13.5, P<0.0001) and 1.4-fold higher than in the bone marrow (241.3 x 10^{-5} ± 15.1, P<0.0001) (**Figure 16**).

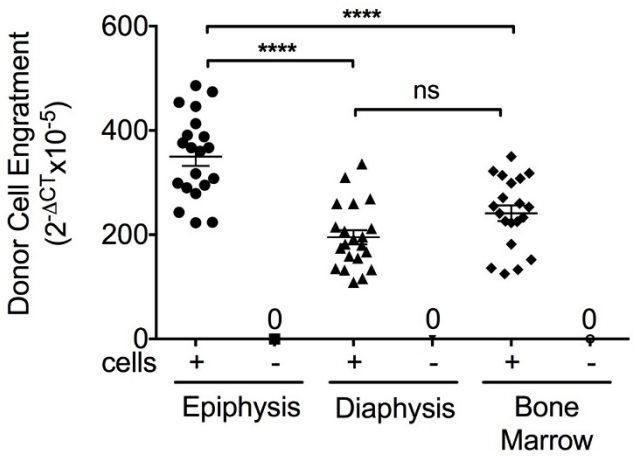


Figure 16. Human AFSCs transplanted into oim mice engraft into the bones.

*Quantitative real time PCR of donor cell engraftment calculated as the $2^{-\Delta\Delta Ct}$ of human specific β-actin normalised to human-mouse non-specific β-actin in the femoral epiphysis, diaphysis and bone marrow (n=20). Data represent mean ± SEM **** P<0001 (One-way ANOVA followed by Bonferroni post hoc test).*

Engraftment of human AFSCs was also assessed also in the soft tissues of 10 transplanted *oim* mice. Cells were not detected in the brain, thymus and spleen of any of the animals analysed. There were low levels of cell engraftment observed in the liver (18.7 x 10^{-5} ± 2.1), lungs (94.7 x 10^{-5} ± 1.6) and kidneys (29.3 x 10^{-5} ± 2.9), all significantly lower than those detected in the bones (P<0.0001) (**Figure 17**).

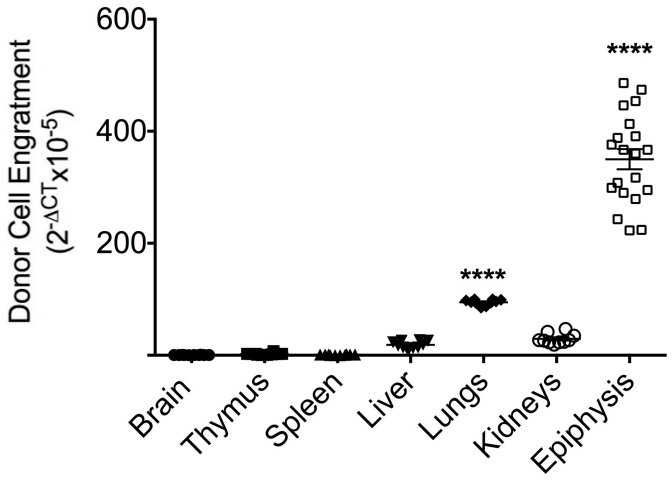


Figure 17. Human AFSCs show low level of multi-organ engraftment in oim mice.

*Quantitative real time PCR of donor cell engraftment calculated as $2^{-\Delta\Delta Ct}$ of human specific β-actin normalised to human-mouse non-specific β-actin in brain, thymus, spleen, liver, lungs and kidneys (n=10), compared to the epiphyses, from figure 14 (n=20). Data represent mean ±SEM **** P<0001 (One-way ANOVA followed by Bonferroni post hoc test).*

In vivo osteogenic differentiation of transplanted human AFSCs was assessed in 8-week old transplanted *oim* tibiae by immunohistochemistry. Differentiated donor cells were visualised using a human-specific anti-osteopontin monoclonal antibody. Osteopontin is a non-collagenous protein, secreted by osteoblasts, which contributes to the formation of the bone ECM. Human osteopontin, absent in non-transplanted *oim* (**Figure 18A** and **D**), was localised mainly in the epiphyseal primary ossification zone (below the growth plate) **(Figure 18C, E** and **F**) and, to a lesser extent, in the cortical region of the tibiae (**Figure 18B**). The osteogenic differentiation of transplanted human AFSCs was evidenced also by the detection of the alpha 2 chain of collagen type I (COL1A2) in transplanted bones (**Figure 19**). *Oim* mice, due to a mutation on the COL1A2 gene do not produce the protein (Chipman et al., 1993).

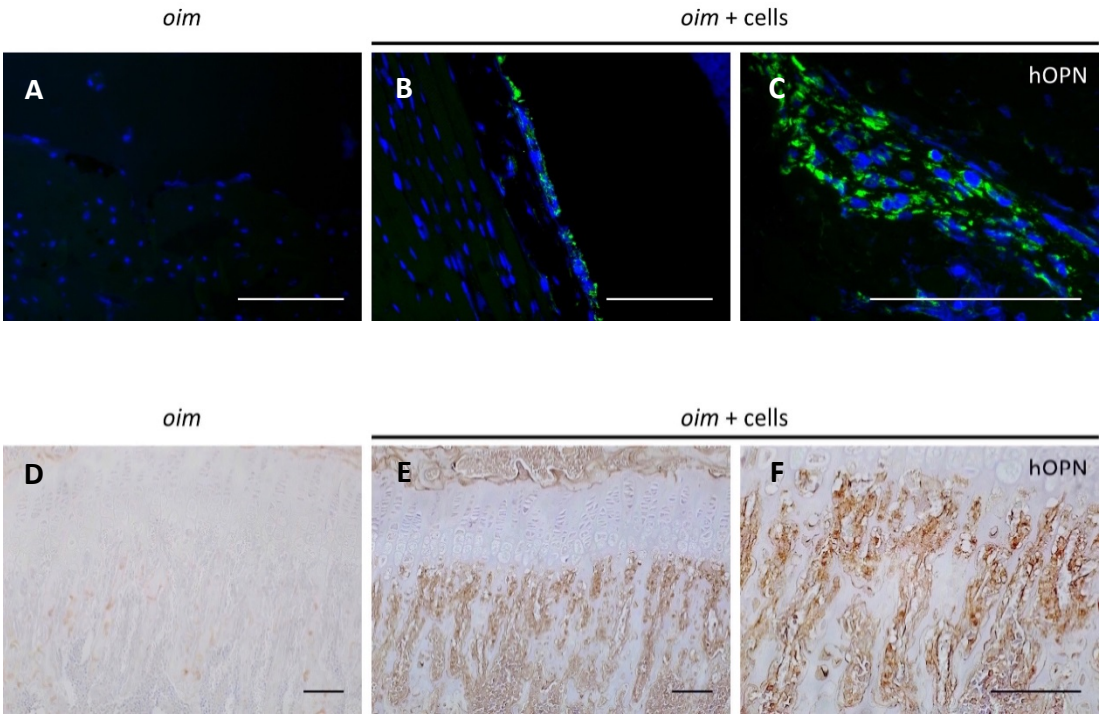


Figure 18. Human AFSCs home to the bones of recipient oim mice and express osteopontin in vivo.

Visualisation of human AFSCs in transplanted oim tibiae with immunofluorescent (A-C) and immunohistochemical (D-F) staining for human osteopontin. Donor cells are visualised in the tibial cortex (B), sub-epiphyseal zone (C) and ossification zone (E-F). An absence of staining was observed in non-transplanted oim tibia (A and D). Scale bars: 100µm.

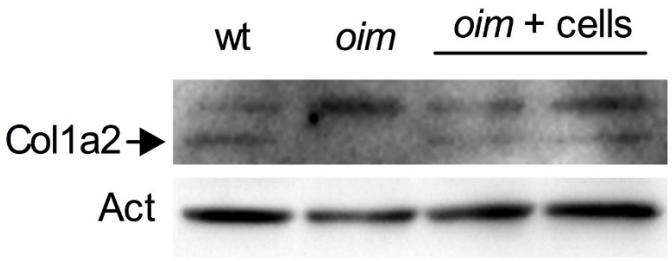


Figure 19. Human AFSCs transplanted into oim mice restore the production of COL1A2.

Western blot of COL1A2 in wild-type (wt), oim and oim transplanted with human AFSCs. β-actin was used as a loading control.

3.3 Discussion

The expression of MSC markers and the ability to differentiate into cells of mesodermal lineages confirmed are consistent with AFSCs being MSCs. Fetal liver MSCs have been used as cell source for *in utero* transplantation in fetuses with severe OI (Götherström et al., 2014). However, their isolation is challenging and relies on termination of pregnancies, which limits their use due to ethical restrictions. Moreover, in a previous study which compared the differentiation potential of a number of fetal and adult MSCs, fetal liver MSCs showed a lower osteogenic potential when cultured in osteogenic permissive media than other fetal MSCs, including fetal blood and fetal bone marrow MSCs (Guillot et al., 2008). Data presented in this chapter support the previous study and suggest that AFSCs grow faster and have a higher *in vitro* osteogenic potential than fetal liver MSCs. Although the experiments described in this thesis were performed using second trimester AFSCs, the comparison with fetal liver MSCs was carried out using first trimester AFSCs, in order to match the gestational age of the fetal liver MSCs available in the laboratory cell bank. Due to a limited number of first trimester samples, the experiments were performed on only one sample per cell type and therefore more samples would be needed to draw any conclusions.

When second trimester AFSCs were transplanted into *oim* mice, cells were detected after 8 weeks in all the 20 mice analysed. Levels of engraftment and distribution of transplanted cells were in accordance with previous studies using the same method of quantification. An estimate of the percentage of engraftment, measured with a standard curve generated previously in the laboratory, suggest a low level of engraftment, around 7%, in line with previous studies (Guillot et al., 2008a; Vanleene et al., 2011; Jones et al., 2014). Transplanted cells preferentially migrated to the bones, supporting the hypothesis that MSCs home to sites of fracture (Granero-Moltó et al., 2009). Moreover, AFSCs underwent *in vivo* osteogenic differentiation and restored the production of the missing collagen chain, again in agreement with previous studies, thus supporting the idea of the amniotic fluid as a promising source of stem cells for cell therapy in OI (Guillot et al., 2008a; Jones et al., 2014).

Chapter 4

Therapeutic effects of AFSCs on *oim* bones

4.1 Introduction and objectives

OI is characterised by generalised osteopenia, caused by the inability of osteoblasts to fully differentiate and produce normal collagen. Moreover, excessive bone resorption, not balanced by new bone formation, leads to loss of bone mass (Forlino et al., 2011). Histomorphometric analyses of iliac bone biopsies from children with OI revealed that bone loss was primarily caused by a 15-27% reduction in the thickness of trabeculae and a 41-57% reduction in the number of trabeculae, compared to age matched healthy children (Rauch et al., 2000). Bone loss leads to frequent long bone fractures, which strongly impact upon the quality of life of OI patients (Trejo and Rauch, 2016). The long bone fracture rate and micro-structural organisation of bones are therefore two of the most important parameters to be assessed for the evaluation of any therapy for OI.

Homozygous *oim* mice resemble the features of severe type III OI, particularly low bone mass, skeletal deformities and multiple fractures (Chipman et al., 1993). *Oim* bones are characterised by fewer trabeculae of reduced thickness, decreased cortical volume and increased porosity compared to wild-type age-matched mice, thus contributing to bone fragility. Moreover, *oim* bones are less strong and elastic, performing worse than controls in biomechanical tests (Bart et al., 2014).

The aim of the work presented in this chapter is to assess whether transplantation of human AFSCs into oim mice ameliorates bone biomechanical properties and bone structure.

The first objective of this chapter is to assess the biomechanical properties of transplanted bones, using the three-point bending test. The second objective is the investigation of the microstructural properties of the trabecular and cortical portions of transplanted tibiae using high–resolution micro computed tomography (micro-CT).

4.2 Results

4.2.1 Transplantation of human AFSCs improves the mechanical properties of *oim* bones

Eight weeks following the transplantation of human AFSCs, bones were isolated from *oim* mice and assessed for fractures along with their biomechanical properties, by other members of the lab. Whilst all the non-transplanted *oim* mice presented at least one long bone fracture, 9/28 transplanted *oim* mice (32.1%) had long bone fractures (Data obtained by Dr. Gemma Jones and Dr. Maximilien Vanleene, **Figure 20**). The fracture incidence in humeri was reduced from 34.6% (18/52 bones) in non-transplanted *oim* to 10.7% (6/56 bones) in transplanted *oim*, which corresponds to a 69% reduction in fracture rate. An 89% reduction was observed in femoral fracture incidence, from 32.7% (17/52) in non-transplanted *oim* to 3.6% (2/56) in transplanted mice; and a 79.2% decrease in tibia fractures, from 17.3% (9/52) in non-transplanted *oim* to 3.6% (2/56) in transplanted mice. The overall fracture incidence in long bones, calculated as the number of fractured tibia, femur, and humeri over the total number of these bones, decreased from 28.2% (44/156 total bones) in non-transplanted *oim* to 5.9% (10/168 total bones) in transplanted *oim*, which corresponds to an overall 79% decrease (Data obtained by Dr. Gemma Jones, not shown).

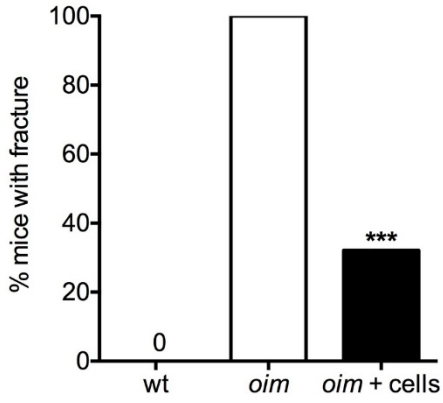


Figure 20. Transplantation of human AFSCs decreases the fracture rate in oim bones.

*Graph showing the percentage of mice with any long bone fractures. N=26 for non-transplanted oim and n=28 for transplanted oim. *** P<0.0001 (Chi-squared test with Yates correction). Data obtained by Dr. Gemma Jones.*

Three-point bending test was used to assess the biomechanical properties of wild-type, *oim* and AFSC-transplanted *oim* femurs (Data obtained by Dr. Maximilien Vanleene). All parameters of bone strength were significantly higher in wild-type than in *oim* femurs (P<0.0001 for each parameter). Human AFSC-transplanted femurs showed a significant increase in all the parameters considered, indicating that stem cell injection increased their overall quality. The total work required to fracture the bone was significantly increased by 141% in transplanted *oim* (**Figure 21A**), whilst the bending stiffness was increased by 64% (**Figure 21B**). Moreover, the ultimate load was increased by 61.8%; and the yield load was increased by 72%. Similarly, work from yield to fracture also significantly increased by 3-fold in transplanted *oim* bones, reflecting an increase in bone plasticity following stem cell injection. The ratio of plastic on total work to fracture (Rp/tW), which describes bone behaviour by assessing plastic deformation, was significantly (2.7-fold) higher in transplanted *oim* bones compared to non-transplanted ones, confirming that transplanted bones were stronger and less brittle (Data not shown, obtained by Dr. Maximilien Vanleene).

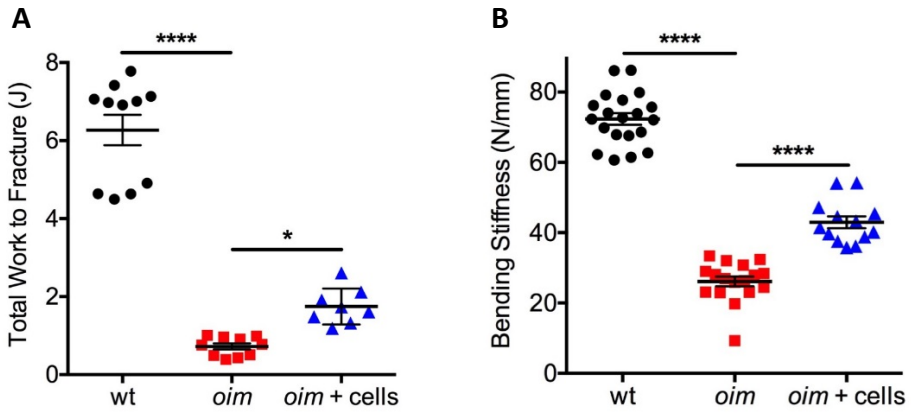


Figure 21. Injection of human AFSCs increased oim bone strength and quality.

*Dot-plot of three-point bending load-deflection curves until fracture. A) total work to fracture, B) bending stiffness. Data represent mean ± SEM * P<0.05, **** P<0.0001 (One-way ANOVA followed by Bonferroni post hoc test). Data obtained by Dr. Maximilien Vanleene.*

The improvement of bone mechanical properties correlated with the numbers of donor cells engrafted. Human AFSC engraftment in the epiphysis of the *oim* femur was positively correlated with total work to fracture (R^2= 0.68, P<0.05, **Figure 22A**) and with bending stiffness (R^2= 0.80, P<0.0001, **Figure 22B**).

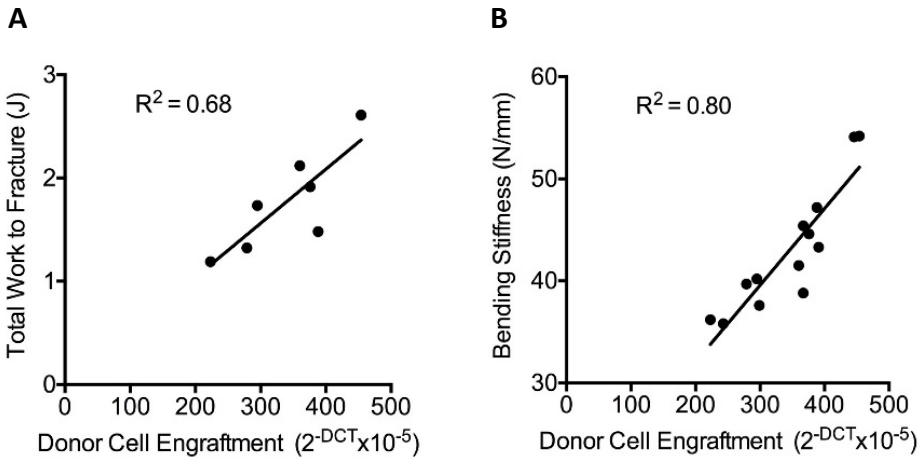


Figure 22. Improvement of oim bone mechanical properties positively correlates with transplanted cell engraftment.

Correlation of transplanted human AFSC engraftment against total work to fracture (A, n=7) and bending stiffness (B n=13) in femurs. Linear line of best fit given. P<0.05 (A) and P<0001 (B).

4.2.2 Transplantation of human AFSCs improves *oim* bone structure

Haematoxylin and eosin staining of histological samples from wild-type, *oim* and transplanted *oim* mice tibiae (n=3 per group), highlighted some structural differences in the epiphyses of wild-type and *oim* bones. Particularly evident was the absence in *oim* tibiae of the numerous and thick trabeculae, characterised by mineralised tissue, present in wild-type mice tibiae (**Figure 23A** arrows and **B**). On the other hand, there were no visible differences in the organisation of the growth plate. The structure of the tissue did not change following transplantation (**Figure 23C**).

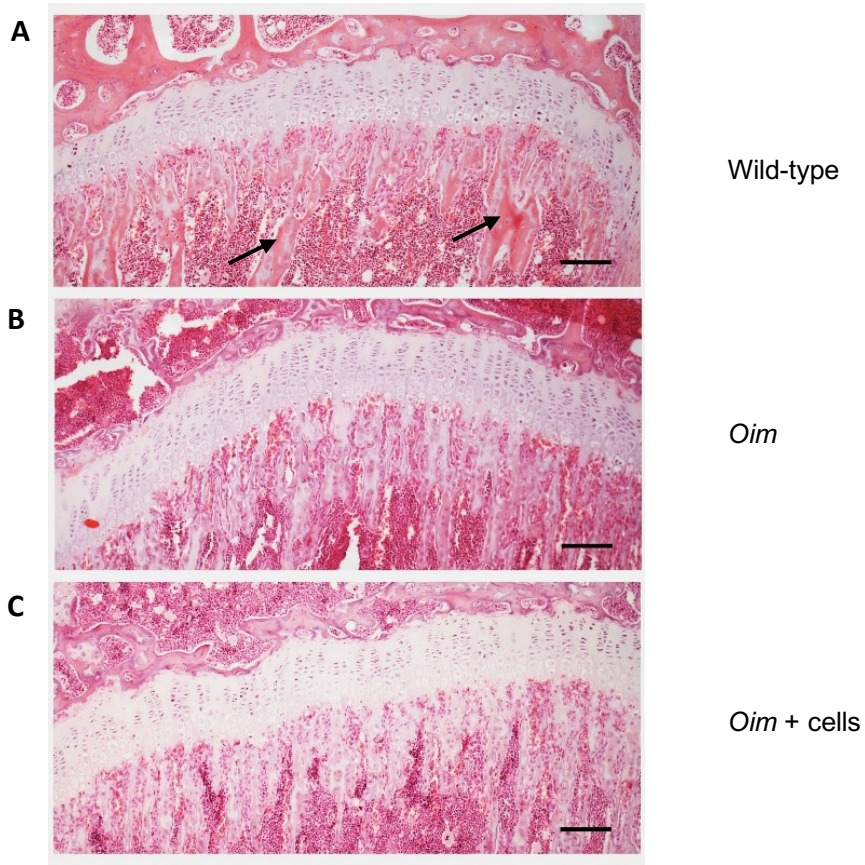


Figure 23. H&E staining of the growth plate of transplanted oim tibiae.

Haematoxylin and eosin staining shows the structure of bone tissue in the growth plate area of A) wild type, B) oim and C) transplanted oim tibiae. Arrow indicates mineralised trabecular bone. Scale bar: 500µm.

To better assess the microarchitecture of trabecular and cortical bone, samples were analysed by micro-computed tomography (micro-CT) (Scans were performed by Dr.

Michelangelo Corcelli). Representative scans of wild-type (n=6), *oim* (n=6) and transplanted *oim* tibiae (n=9) are shown (**Figure 24**).

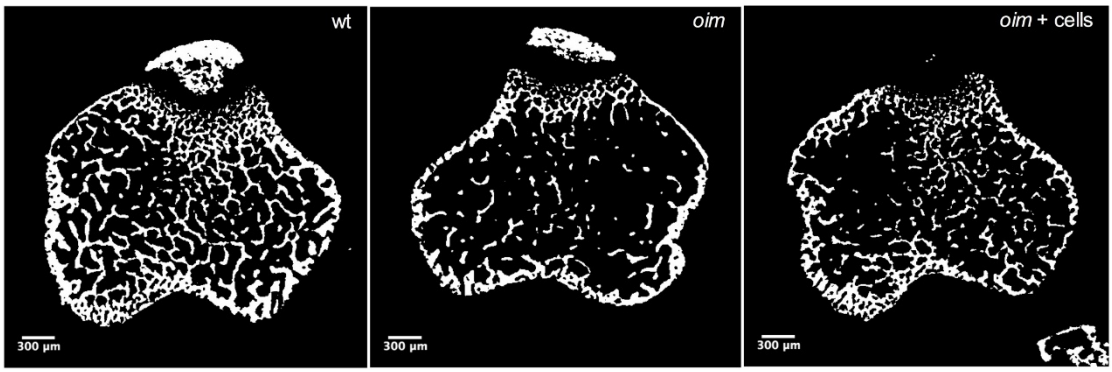


Figure 24. Micro-CT analysis of transplanted oim tibiae.

Representative micro-CT images of trabecular bone cross-sections at 0.25 mm below the tibial growth plate, obtained for 8-week-old wild-type, non-transplanted oim and transplanted oim. Scale bar: 300µm

Trabecular thickness and trabecular number were significantly higher in wild-type than in *oim* bones (0.038 ± 0.001 mm vs. 0.030 ± 0.001 mm, P<0.01; and 3.18 ± 0.15 mm^{-1} vs. 1.27 ± 0.19 mm^{-1}, P<0.001, respectively) and were not improved by transplantation (0.031 ± 0.001 mm and 1.670 ± 0.22mm^{-1}, respectively) (**Figure 25A** and **B**). Similarly, trabecular separation, which resulted lower in wild-type than in *oim* tibiae (0.17 ± 0.01 mm vs. 0.256 ± 0.019 mm, P<0.01), was unchanged in transplanted *oim* (0.29 ± 0.03 mm) (**Figure 25C**). Bone volume/total volume, which was significantly higher in wild-type than in *oim* bones (12.25 ± 0.63% vs. 3.94 ± 0.67%, P<0.0001), was slightly increased in transplanted *oim* (5.22 ± 0.67%) albeit not to a significant degree (**Figure 25D**). Trabecular bone pattern factor, which is a unit of measure of trabecular connectivity (Hahn et al., 1992) was significantly lower in wild-type than in non-transplanted *oim* mice (27.34 ± 0.94 vs 52.80 ± 2.60, P<0.0001), indicating a better architectural trabecular organisation in wild-type mice. The trabecular pattern factor was significantly lowered by 19% in transplanted *oim* mice tibiae (42.79 ± 2.88, P<0.05), indicating that stem cell transplantation did not increase the production of bone tissue, but rather improved the orientation and connectivity of trabeculae (**Figure 25E**).

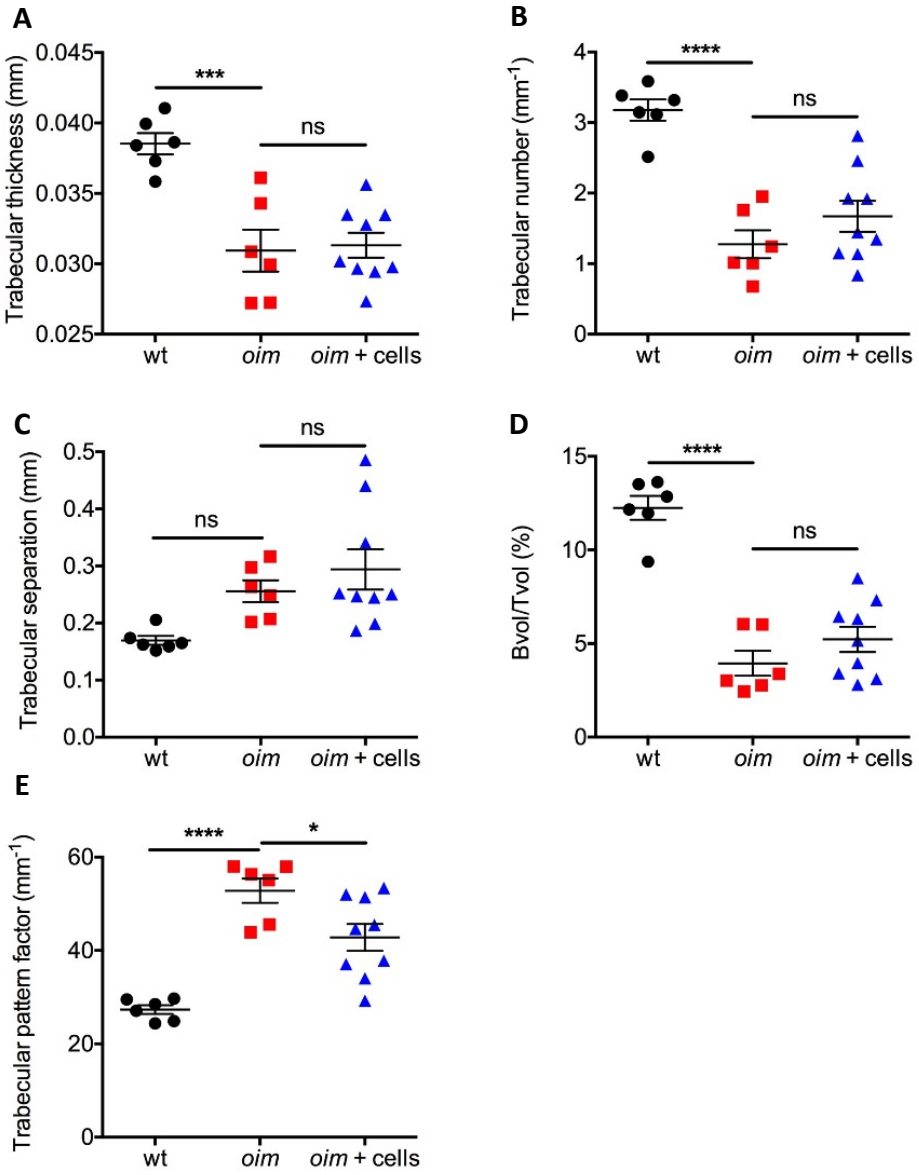


Figure 25. AFSC transplantation improves trabecular connectivity in oim mice without increasing bone volume.

*Dot plot of micro-CT trabecular morphological parameters, in wild-type (n=6), non-transplanted oim (n=6) and transplanted oim (n=9) mice: Trabecular thickness (A), Trabecular number (B), Trabecular separation (C), Bone volume/total volume (D), Trabecular pattern factor (E). Data represent mean ± SEM * P<0.05, *** P<0.001, **** P<0.0001 (One-way ANOVA followed by Bonferroni post hoc test).*

Following calibration with phantoms of known calcium hydroxyapatite (CaHA) content, bone mineral density (BMD) and tissue mineral density (TMD) were measured in wild-type, *oim* and transplanted *oim* tibiae. Both BMD and TMD indicate the volumetric density of CaHA in a biological tissue in g/cm^3. BMD relates to the amount of bone in a mixture of hard and soft tissue and is used to assess mineral density in trabecular tissue. TMD, on the other hand, is used to measure mineral density in cortical bone and refers to the material density of the bone, without taking into account any soft tissue (Bouxsein et al., 2010). BMD was measured in the trabecular portion of the tibiae. Wild-type bones had a higher BMD than *oim* ones (0.13 ± 0.01 g/cm^3 vs 0.03 ± 0.01 g/cm^3, P<0.0001) and this parameter was not significantly altered following transplantation with human AFSCs (0.06 ± 0.01 g/cm^3) (**Figure 26A**). TMD was measured in the cortical part of the tibiae. Wild-type and *oim* tibiae did not differ in TMD (1.02 ± 0.04 g/cm^3 vs 1.02 ± 0.01 g/cm^3), however transplantation significantly increased the mineral content in the cortex of the bone (1.10 ± 0.01 g/cm^3, P<0.01) (**Figure 26B**).

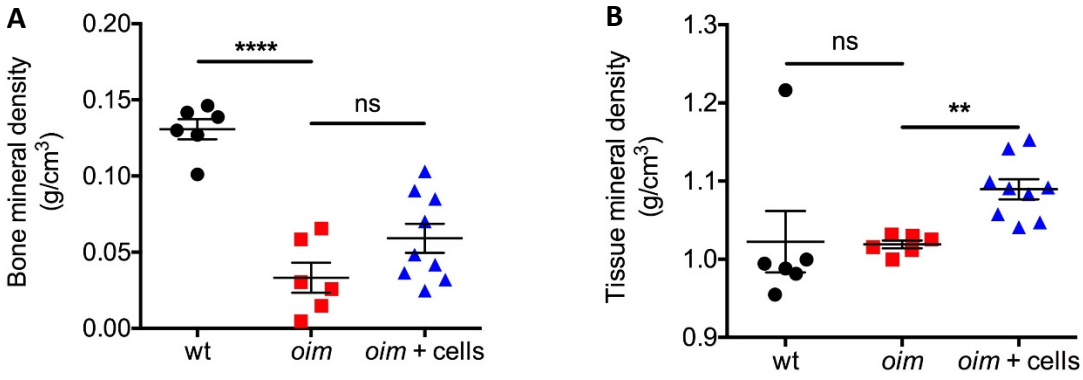


Figure 26. AFSC transplantation increased the cortical mineral content of oim tibiae.

*Dot-plot of mineral density measures of wild-type (n=6), oim (n=6) and AFSC-transplanted oim tibiae (n=9). A) Bone mineral density measured in the trabecular region. B) Tissue mineral density measured in the cortical region. Data represent mean ± SEM ** P<0.01, **** P<0.0001 (One-way ANOVA followed by Bonferroni post hoc test).*

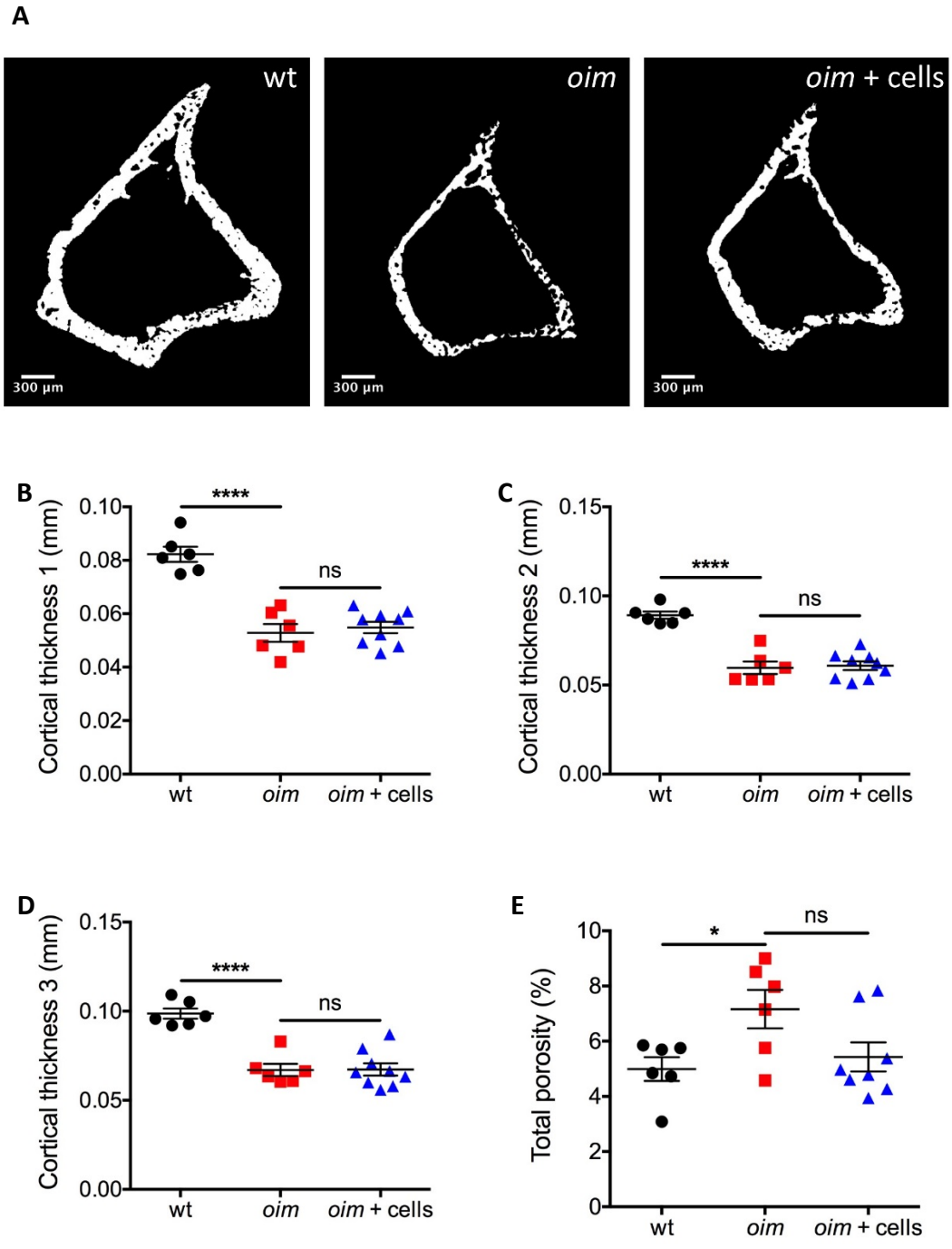


Figure 27. AFSC transplantation did not improve cortical thickness and bone porosity in oim tibiae.

*A) Representative micro-CT images of cortical bone cross-sections at 1.75 mm below the growth plate, obtained for 8-week-old wild-type, oim and AFSC-transplanted oim. Dot plot of micro-CT cortical morphological parameters, in wild-type (n=6), oim (n=6) and transplanted oim (n=9) mice. Cortical thickness measured at 1.75 mm (B), 2.25 mm (C) and 2.75 mm (D) below the growth plate. Total cortical porosity of the cortex (E). Data represent mean ± SEM * P<0.05, **** P<0.0001 (One-way ANOVA followed by Bonferroni post hoc test).*

Cortical thickness was significantly decreased in *oim* compared to wild-type bones and transplantation did not cause any change. Additionally, porosity was significantly increased in *oim* compared to wild-type bones and this parameter was not improved following transplantation of human AFSCs either. Representative micro-CT sections of cortical bone are shown in **Figure 27A**. Cortical thicknesses measured in the three different areas along the length of the tibia were 0.082 ± 0.002 mm, 0.089 ± 0.002 mm and 0.098 ± 0.003 mm in wild-type, 0.052 ± 0.003 mm, 0.059 ± 0.003 mm and 0.067 ± 0.003 mm in *oim* and 0.054 ± 0.002 mm, 0.060 ± 0.002 mm and 0.067 ± 0.003 mm in transplanted *oim bones* (**Figure 27B-D**). Cortical porosity was significantly higher in *oim* compared to wild-type tibiae (1.20 ± 0.16 % vs 0.78 ± 0.07%, $P<0.05$) and remained unchanged after transplantation (1.04 ± 0.13%) (**Figure 27E**).

MicroCT was also used to quantify the area of primary ossification, which refers to the area below the growth plate where cartilage starts to mineralise. Representative images of primary ossification in wild-type (n=6), *oim* (n=6) and transplanted *oim* (n=9) are shown in **Figure 28A**. *Oim* tibiae had a much thicker primary ossification area (0.29 ± 0.03 mm vs 0.16 ± 0.01 mm, $P<0.001$), which decreased significantly following transplantation with human AFSCs (0.20 ± 0.01 mm, $P<0.05$) (**Figure 28B**).

Subchondral bone is located below the joint cartilage and plays an important role in absorbing stress. Its structural properties were evaluated by micro-CT for wild-type (n=6), *oim* (n=6) and *oim* transplanted with human AFSCs (n=9). All parameters tested were worse in *oim* compared to wild-type bones and were not changed following transplantation. In particular, Bvol/Tvol percentage in wild type was 23.88 ± 1.21% vs. 13.21 ± 1.56% in *oim*, $P<0.0001$, vs. 15.15 ± 0.52% in transplanted *oim* (**Figure 29A**). Trabecular pattern factor in wild-type was -19.00 ± 2.40 mm^{-1} vs. 11.05 ± 5.69 mm^{-1} in *oim* ($P<0.001$) vs. 2.63 ± 1.71 mm^{-1} in transplanted *oim* (**Figure 29B**). Similarly, trabecular thickness in wild-type was 0.048 ± 0.001 mm vs. 0.038 ± 0.002 mm in *oim* ($P<0.01$) vs. 0.039 ± 0.001 mm in transplanted *oim* (**Figure 29C**) and trabecular number in wild-type was 4.95 ± 0.12 mm^{-1} vs. 3.40 ± 0.24 mm^{-1} in *oim* ($P<0.01$) vs. 3.85 ± 0.14 mm^{-1} following transplantation (**Figure 29D**).

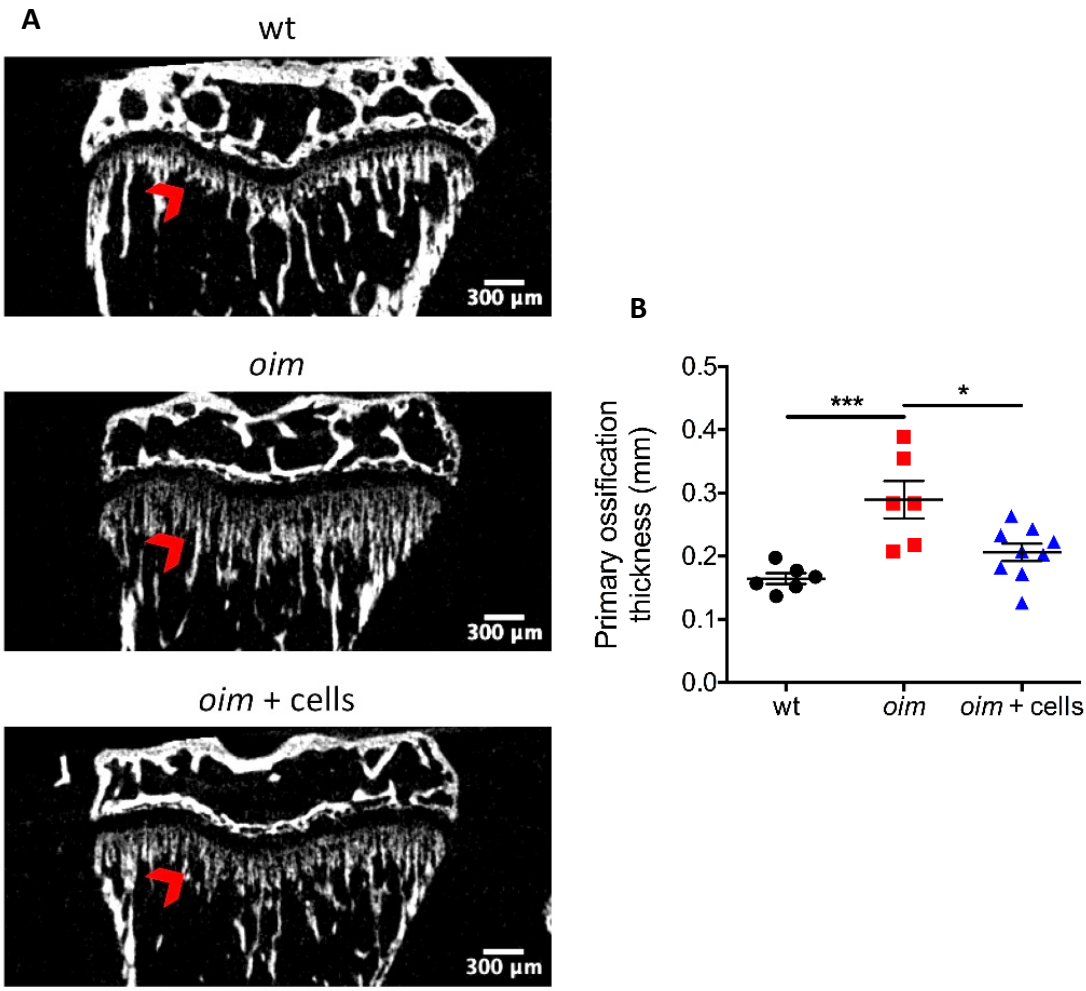


Figure 28. AFSC transplantation decreased immature ossification in oim tibiae.

*A) Representative micro-CT images of primary ossification, obtained for 8-week-old wild-type (n=6), non-transplanted oim (n=6) and transplanted oim (n=9). B) Dot plot of thickness of the primary ossification area of wild-type, oim and transplanted oim. Data represent mean ± SEM * P<0.05, *** P<0.001 (One-way ANOVA followed by Bonferroni pots hoc test).*

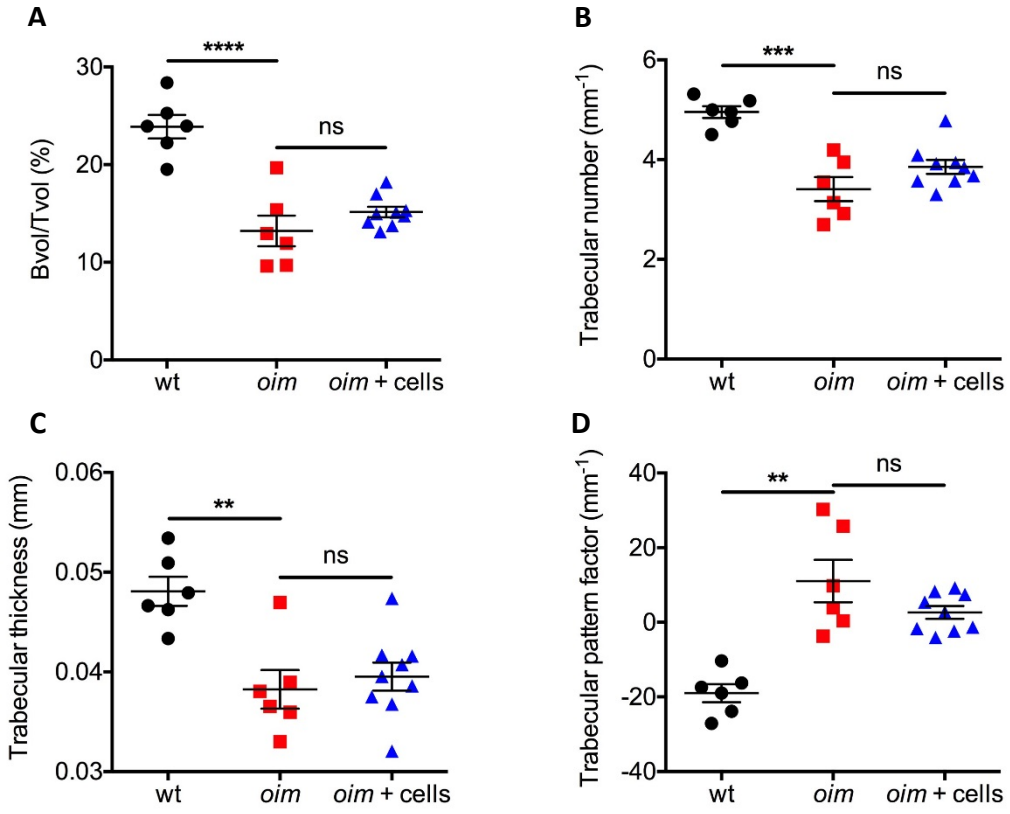


Figure 29. Transplantation of human AFSCs did not affect subchondral bone in oim mice.

*Dot plot of subchondral bone micro-CT morphological parameters, in wild-type (n=6), non-transplanted oim (n=6) and transplanted oim (n=9) mic tibiae. A) bone volume/total volume, B) trabecular pattern factor, C) trabecular thickness and D) trabecular number. Data represent mean ± SEM ** P<0.01, *** P<0.001 and **** P<0.0001 (One-way ANOVA followed by Bonferroni post hoc test).*

4.3 Discussion

Despite the overall low level of AFSC engraftment, transplantation caused a substantial decrease in fracture rate and improved bone biomechanical properties. Moreover, the increase in bone strength and bending stiffness positively correlated with the number of cells engrafted in bones. Other studies have also reported significant decreases in the number of fractures despite low levels of cellular engraftment, possibly due to the paracrine effect of donor stem cells on resident defective cells (Guillot et al., 2008a; Vanleene et al., 2011; Jones et al., 2014).

In this study, the 79% reduction in long bone fracture rate was not accompanied by changes in the total volume of bone tissue, the number or the thickness of the trabeculae. The only parameter that was significantly improved following transplantation was the trabecular pattern factor, an index of trabecular connectivity. This parameter is a widely used index of the relative concavity of trabecular bone surface. Surface concavity indicates the presence of connected structures, while surface convexity relates to isolated and disconnected structures (Hahn et al., 1992). This is in contrast with previous work, carried out in the Guillot lab, showing that transplantation of human chorionic stem cells into *oim* mice increased bone volume and led to higher ductility of the bones, whilst bone strength and stiffness remained unchanged (Jones et al., 2014). This suggests that cells from the placenta and the amniotic fluid improve the OI phenotype by different mechanisms.

One of the main limitations of this and previous preclinical studies for cell therapy in OI is the absence of longer time points. The longest follow-up analysis in a mouse model transplanted with stem cells was performed at 12 weeks post-transplantation and there are no data available on the long-term effects of transplantation (Guillot et al., 2008). Clinical data from OI children transplanted with MSCs demonstrated that the beneficial effects of stem cell administration, including boosts in growth and reduction of fracture number, were limited to the first few months after treatment and repeated transplantations were needed (Horwitz et al., 2002; Götherström et al., 2014). Longer term investigations will therefore be necessary before cell therapy approaches in OI are translated into clinical practice.

Chapter 5

Therapeutic effects of AFSCs on the bone cells of *oim* mice

5.1 Introduction and objectives

Data from this study and others show that MSCs, when transplanted into the *oim* model, are able to home to the bones, possibly due to their attraction to inflammatory cytokines, and undergo osteogenic differentiation, depositing normal type 1 collagen (Wang et al., 2006; Guillot et al., 2008a; Li et al., 2010a; Jones et al., 2014). In addition to this direct contribution to tissue repair, an indirect effect of MSCs on resident cells, through the release of paracrine factors, has been hypothesised as a mechanism of action in MSC-based therapies (Prockop, 2017). MSCs have been shown to release a wide variety of molecules, including growth factors and anti-inflammatory cytokines (Doorn et al., 2012).

The *oim* model is characterised by frequent bone fractures, which lead to the release of pro-inflammatory cytokines and, at the cellular level, by impaired osteoblasts that are unable to fully differentiate (Li et al., 2010b). Therefore, the paracrine effect of MSCs could explain, at least in part, the significant improvement in disease phenotype observed here despite the low level of cell engraftment.

Moreover, a recent study on a mouse model of recessive OI suggested that excessive TGF-β signalling contributes to the pathogenesis of OI. The authors show that the defective OI extracellular matrix is unable to retain the inactive form of TGF-β. This leads to the abnormal release of the active form, which has been linked to the inhibition of osteoblast differentiation (Alliston et al., 2001; Grafe et al., 2014).

The aim of this chapter is to assess whether transplanted human AFSCs exert their *in vivo* therapeutic effect via a paracrine mechanism.

The objective of this chapter is the investigation of the *in vivo* effects of AFSC transplantation on *oim* resident cells, in terms of gene expression. In order to investigate the indirect contribution of donor cells, the expression of *oim* endogenous genes involved in skeletal development, bone turnover, osteoblast differentiation, inflammation and TGF-β signalling was assessed in transplanted and non-transplanted *oim* bones.

5.2 Results

5.2.1 Transplantation of human AFSCs stimulates endogenous osteogenesis in oim mice

In order to investigate the potential paracrine effect of transplanted human AFSCs on resident *oim* osteoblasts, the expression levels of a panel of endogenous mouse genes involved in skeletal development were assessed. A mouse-specific gene array was used to evaluate the endogenous expression of the gene panel in the femoral epiphysis of *oim* (n=2) and AFSC-transplanted *oim* (n=2). An overall upregulation of the genes involved in skeletal development, bone mineral metabolism, and ECM development was observed, whilst no changes were found in the expression of genes involved in the regulation of the cell cycle. In particular, genes involved in bone mineralisation such as ameloblastin (*Ambn)* and enamelin (*Enam*) (Stakkestad et al., 2017), were upregulated by 3.5 and 3.1-fold respectively. Transplanted *oim* mice also showed an 8.4 fold increase in the expression of SRY-Box 9 (*Sox9*), a gene involved in mesenchymal condensation and cartilage development (Bi et al., 2006; Zhang et al., 2017). Dentin matrix protein 1 (*Dmp1*) and *Phex,* genes expressed by osteoblasts which govern the mineralisation of bone via the FGFR pathway (Martin et al., 2011; Ichikawa et al., 2017), showed an up-regulation of 10.6 and 4.6-fold respectively. Human AFSC transplantation also stimulated the production of collagen type I by resident cells, as shown by the 5.5-fold upregulation.

The gene array also revealed an upregulation of bone morphogenetic proteins *(Bmp)* 2, 4, 5 and 6 (2.3, 3.4, 3.5 and 4.7-fold respectively) (**Figure 30A**). BMP proteins are part of the TGF-β superfamily and are key regulators of bone and cartilage development (Grimaud et al., 2002; Majidinia and Yousefi, 2017). BMP2 in particular acts as an osteoinductive agent and is involved in the commitment to the osteogenic lineage (Urist, 1965; Nguyen et al., 2017). AFSC-transplantation also caused an upregulation of genes involved in ECM formation. *Serpinh1*, a gene responsible for collagen type I folding (Masago et al., 2012), showed a 8.8-fold increase in expression. Finally, cartilage oligomeric matrix protein (*Comp*), gene involved in chondrocyte

organisation (Haleem-Smith et al., 2012), was upregulated by 3.9-fold (**Figure 30B**). Transplantation did not influence the expression of endogenous genes involved in cell proliferation (**Figure 30C**).

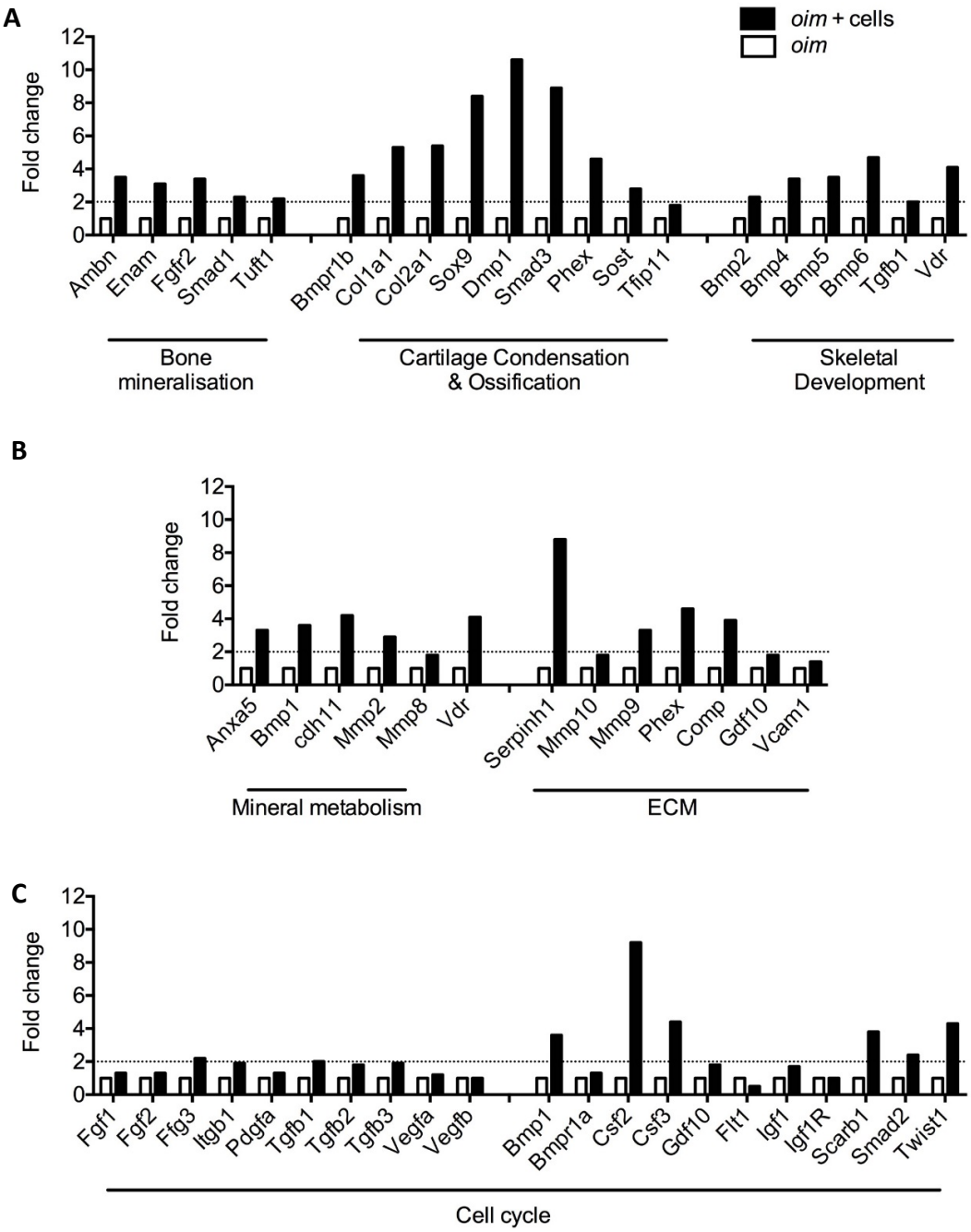


Figure 30. AFSC transplantation upregulated endogenous genes of oim mice involved in osteogenesis.

Graph to show fold changes in the expression of genes involved in A) osteogenesis, B) ECM formation and C) cell proliferation in the femoral epiphysis of human AFSC-transplanted oim compared to non-transplanted oim mice (n=*2) (2-fold change selected as threshold cut-off).*

Importantly, an upregulation of genes involved in osteoblast differentiation and maturation was observed. Runt-related transcription factor 2 (*Runx2*), which plays a pivotal role in the differentiation of MSCs into osteoblasts (Ducy et al., 1997; Komori et al., 1997), showed a 11.3-fold increase in expression following transplantation. Moreover, osteocalcin (*Bglap*), a marker of mature osteoblasts (Ryoo et al., 1997), increased its expression of 3-fold (**Figure 31**).

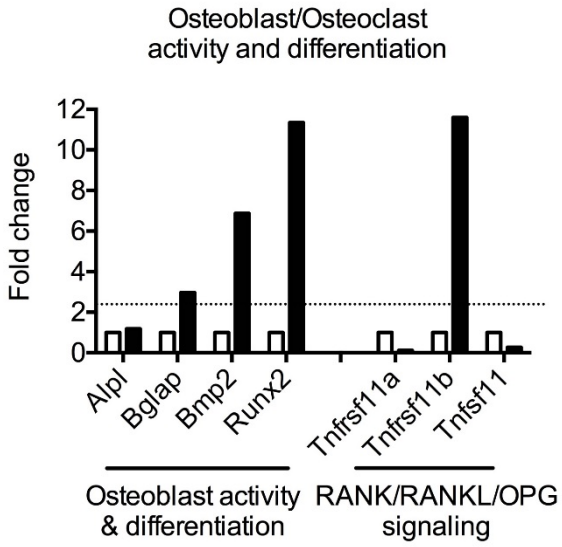


Figure 31. AFSC transplantation upregulated endogenous genes of oim mice involved in osteoblast differentiation.

Graph to show fold changes in the expression of genes involved in osteoblast development and differentiation in the femoral epiphysis of human AFSC-transplanted oim, compared to non-transplanted oim mice (n=2) (2-fold change selected as threshold cut-off).

Gene array analyses also showed that transplantation with human AFSCs decreased the expression of genes involved in osteoclast activity. RANK ligand (*Tnfsf11*, RANKL), is produced by pre-osteoblasts and, by binding to its receptor RANK (*Tnfrsf11a*) on osteoclasts, stimulates osteoclast differentiation. OPG (*Tnfrsf11b*, Osteoprotegerin) is instead a decoy receptor for RANKL, produced by differentiated osteoblasts. A high RANKL/OPG ratio is therefore present in actively resorbing bones, whereas a low ratio indicates that the bone turnover status is shifted toward bone formation (Teitelbaum, 2000).

RANKL and OPG expression was further assessed by quantitative real-time PCR in *oim* (n=7) and transplanted *oim* (n=12). RANKL was significantly down-regulated following transplantation (P<0.01) (**Figure 32A**). OPG was slightly up-regulated following transplantation, albeit not statistically significant. (**Figure 32B**).

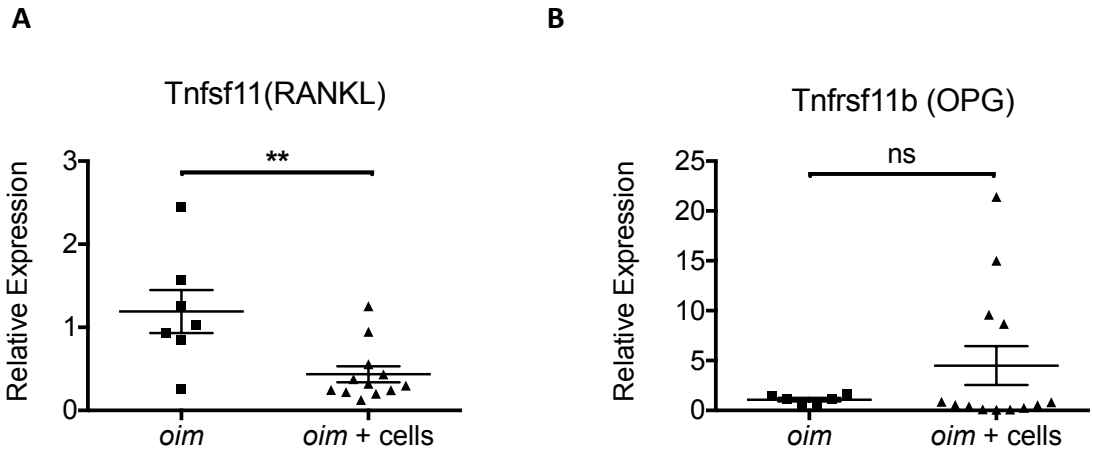


Figure 32. AFSC transplantation lowered the RANKL expression in oim mice.

*Quantitative real time PCR of expression of A) RANK ligand and B) Osteoprotegerin in oim (n=7) and human AFSC-transplanted oim (n=12). Data represent mean ± SEM ** P<0.01 (Student's t-test).*

Osteocalcin expression in wild-type (n=2), *oim* (n=5) and transplanted *oim* (n=8) femurs was assessed at the protein level by western blot analysis. *Oim* mice had significantly lower levels of osteocalcin compared to wild-type age matched controls (Bglap/Actin ratio: 11.76 ± 0.73 in wild-type vs 4.96 ± 1.17 in *oim* P<0.05). Following transplantation, levels of osteocalcin appear to be increased, although not reaching significance, due to the high variability between animals (9.51 ± 2.10) (**Figure 33**).

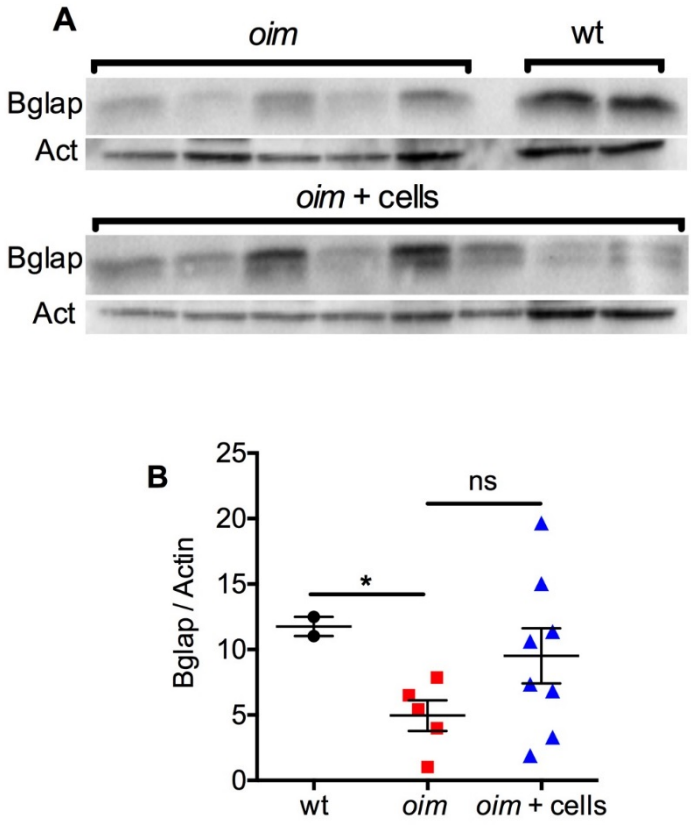


Figure 33. AFSC transplantation increased osteocalcin levels in oim mice.

*A) Western blot analysis of osteocalcin in wild-type (n=2), oim (n=5) and human AFSC-transplanted oim (n=8) femurs. B) Quantification of western blot after normalisation to ß-actin. Data represent mean ± SEM * P<0.05 (One-way ANOVA followed by Bonferroni post hoc test).*

5.2.2 Transplantation of human AFSCs does not affect the inflammatory status of *oim* bones but downregulates TGF-β signalling

The expression of endogenous genes involved in inflammation was assessed in *oim* (n=7) and transplanted *oim* (n=12) femurs. Expression levels of TNFα (**Figure 34A**) and NFκB (**Figure 34B**) did not differ significantly in transplanted *oim* compared to *oim*.

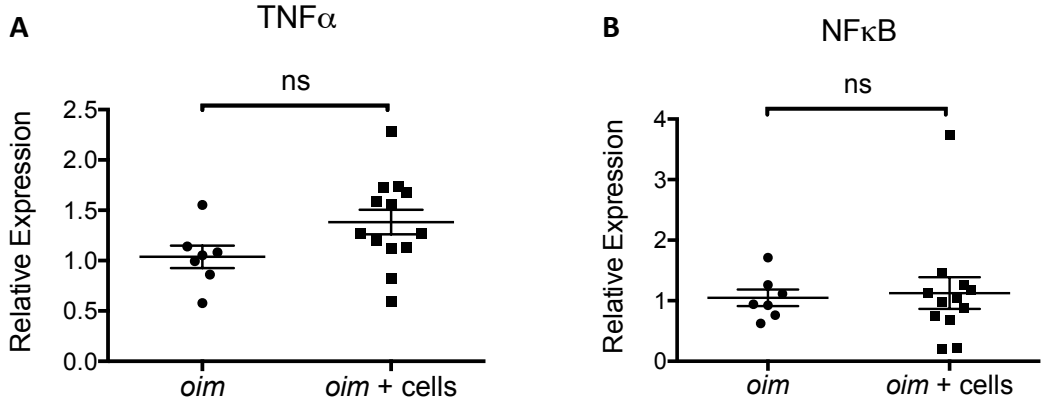


Figure 34. AFSC transplantation did not affect endogenous expression of oim mice genes involved in inflammation.

*Quantitative real time PCR of expression of A) TNFα and B) NF*κB *in oim (n=7) and human AFSC-transplanted oim (n=12). Data represent mean ± SEM (Student's t-test).*

Increased TGF-β signalling, due to excessive levels of active TGF-β being released from the abnormal OI ECM, has recently been linked to the pathogenesis of OI in mouse models (Grafe et al., 2014b). In order to investigate the status of TGF-β signalling following transplantation, the expression of endogenous plasminogen activator inhibitor-1 (*Serpine1)* and cyclin-dependent kinase inhibitor 1a (*Cdkn1a)*, two known TGF-β targets, was assessed by quantitative RT-PCR. Transplantation with human AFSCs significantly decreased the expression of *Serpine1* ($P<0.01$) (**Figure 35A**).

The expression of *Cdkn1a* was slightly reduced in transplanted *oim* compared to non-transplanted *oim* mice, albeit not statistically significant (**Figure 35B**).

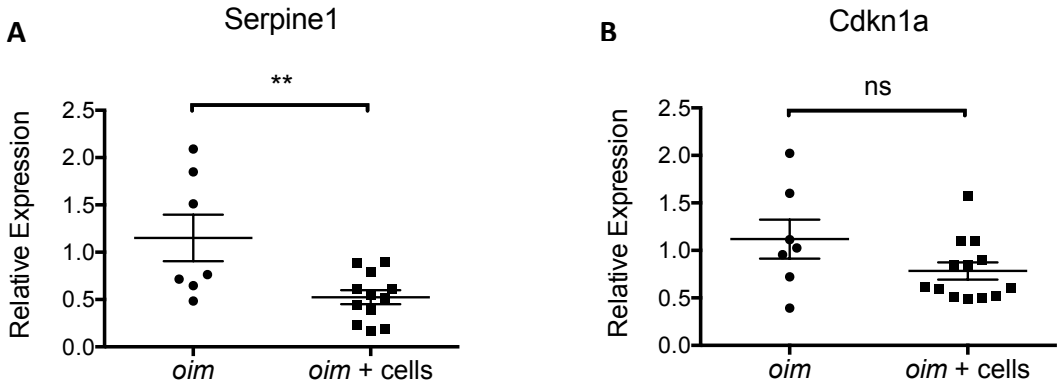


Figure 35. Transplantation of human AFSCs decreased TGF-β signalling in oim mice.

*Quantitative real time PCR of expression of TGF-β targets A) Serpine1 and B) Cdkn1a in oim (n=7) and human AFSC-transplanted oim (n=12). Data represent mean ± SEM ** P<0.01 (Student's t-test).*

5.3 Discussion

Data from this study, as well as others, indicate that transplantation of human MSCs improves the phenotype of *oim* mice, partly due to the cell replacement paradigm, in which cells differentiate *in vivo* and replace damaged cells. Nevertheless, the low level of engraftment, together with the lack of increase in bone volume following transplantation and the up-regulation of endogenous genes involved in bone development, suggest that other mechanisms might be involved (Jones et al., 2014). Results presented in this chapter, although not conclusive due to the small number of samples used for the gene arrays (n=2), are consistent with the paracrine effect theory. This theory hypothesises that transplanted cells do not exert their beneficial function by directly contributing to the regeneration of the injured tissue, differentiating and replacing damaged cells, but rather by secreting soluble factors that modulate resident cells (Prockop, 2017). Osteoblasts in the *oim* model, as well as in the Brtl IV model, are unable to fully differentiate and express lower levels of osteogenic genes, including Runx2 and type I collagen (Li et al., 2010b; Gioia et al., 2012). Endogenous gene expression analyses suggest that AFSC transplantation promotes the differentiation of *oim* osteoblasts, possibly by a paracrine effect. Moreover, transplanted mice showed a lower expression of RANKL in bones, compared to non-transplanted *oim* mice. This suggests that transplantation decreased osteoclastogenesis and led to lower rates of bone turnover. On the other hand, the inflammatory status of bones before and after transplantation did not change, as shown by the expression of inflammatory genes TNFα and NFκB.

From these data, a hypothesis for the mechanism of action of transplanted AFSCs has been developed and will be the focus of future work in the group (**Figure 36**). At a cellular level, OI in the *oim* model is manifested through the inability of pre-osteoblasts to produce the α2 chain of collagen type I, leading to the formation of homotrimeric collagen molecules and of a disorganised extracellular matrix. On the other hand, *oim* osteoblasts fail to fully differentiate and remain at a pre-osteoblast stage, which has been reported to promote osteoclastogenesis, thus unbalancing bone turnover (Martin et al., 2011b; Gioia et al., 2012). More recently, a study

showed that an up-regulated TGF-β signalling is present in OI, due to the excessive release of TGF-β from the ECM, and further contributes to the inhibition of osteoblast differentiation (Grafe et al., 2014).

Thus, transplantation of human AFSCs may improve the disease phenotype by two mechanisms. Firstly, AFSCs differentiate into osteoblasts *in vivo* and produce the missing Col1α2 chain, thus normalising the defective ECM and restoring TGF-β sequestration. Moreover, AFSCs may produce trophic factors that stimulate the differentiation of osteoblasts. Moreover, mosaic carriers that have a small number of healthy osteoblasts producing the correct collagen chains show little or no symptoms, suggesting that a chimeric matrix, even with little engraftment, can have therapeutic effects (Cabral and Marini, 2004).

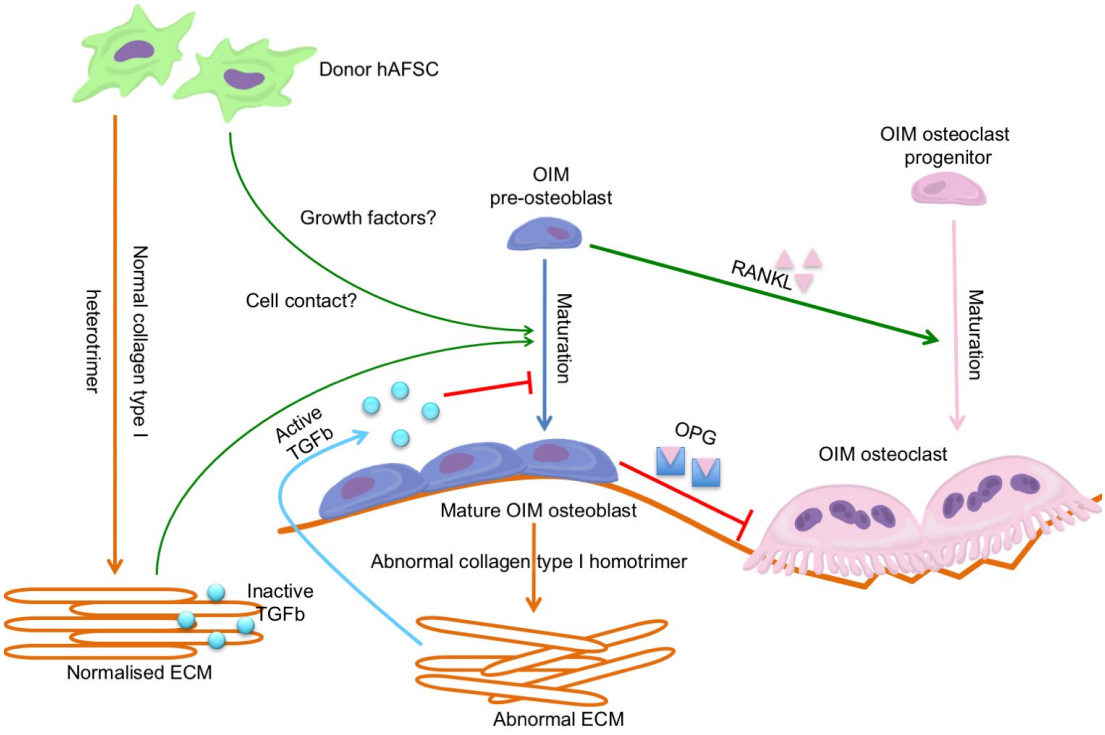


Figure 36. Model of mechanism of action of AFSCs in the oim model.

Oim osteoblasts (blue) produce abnormal collagen type 1 (orange). The defective ECM releases excessive levels of TGF-β which inhibits osteoblast differentiation. Pre-osteoblasts promote osteoclastogenesis by the release of RANKL (pink). Transplanted AFSC normalise the ECM and restoring TGF-β sequestration. On the other hand, paracrine factors produced by AFSCs may promote the differentiation of resident oim osteoblasts.

Chapter 6

In vitro investigation of the mechanism of action of AFSCs on *oim* osteoblasts

6.1 Introduction and objectives

In vivo mechanisms that lead to therapeutic benefits in the *oim* model, following transplantation of MSCs, are complex and difficult to study. Donor cells may act in different ways, both direct and indirect, and many signalling pathways are thought to be involved. In order to better understand the impact of MSCs on resident cells, *in vitro* modelling can be useful, since it allows to isolate events in a controlled environment, without the influence of other cell types present in the *in vivo* setting. Moreover, *in vitro* modelling and co-cultures between relevant cell types have been essential in the elucidation of the aetiology of diseases, including OI (Martin et al., 2011; Gioia et al., 2012).

The main aim of this chapter is to investigate *in vitro* the effect of AFSCs on resident osteoblasts, in order to better understand the mechanism of action of donor cells. The second aim is to establish a protocol for the isolation and differentiation of human fetal osteoblasts to be used to model bone formation *in vitro*.

The first objective of this chapter is to recapitulate *in vitro* the effects of human AFSCs seen on osteoblasts *in vivo*. The effect of AFSC-conditioned medium was investigated, with AFSCs either in their naïve state or following pre-conditioning with inflammatory factors, in order to better mimic the inflammatory environment of OI bones.

Protocols have been developed for the isolation and differentiation of murine osteoblasts, which can be obtained in large numbers and are able to form bone efficiently when cultured in osteogenic permissive medium (Orriss et al., 2012). Murine osteoblasts, however, have a limited proliferation capacity and are unable to differentiate following repeated passaging or freezing.

There have been a number of attempts to isolate human osteoblasts, both from adult and fetal tissues. Adult-derived osteoblasts can be obtained from hip-replacement surgical waste, however they are usually derived from older patients and have low growth and differentiation ability (D'Ippolito et al., 1999; Gallagher, 2003). More recently, fetal tissues have been investigated as a better alternative for the isolation of MSCs and pre-osteoblasts (Gothard et al., 2015).

The second objective of this chapter is the optimisation of an enzymatic digestion protocol for the efficient isolation and differentiation of human fetal osteoblasts, derived from calvarial bone tissue obtained from terminated pregnancies.

6.2 Results

6.2.1 Co-culture of human AFSCs and oim osteoblasts

In order to recapitulate *in vitro* the effect of human AFSCs on *oim* osteoblasts, calvaria from *oim* 7-day-old pups were dissected and osteoblasts were isolated. Two different methods were used: explant culture and enzymatic digestion with trypsin and collagenase, as described in (Orriss et al., 2012). *Oim* osteoblasts derived by the digestion protocol were obtained in higher numbers and faster than by explant outgrowth and were therefore used for the co-culture experiments (**Figure 37**).

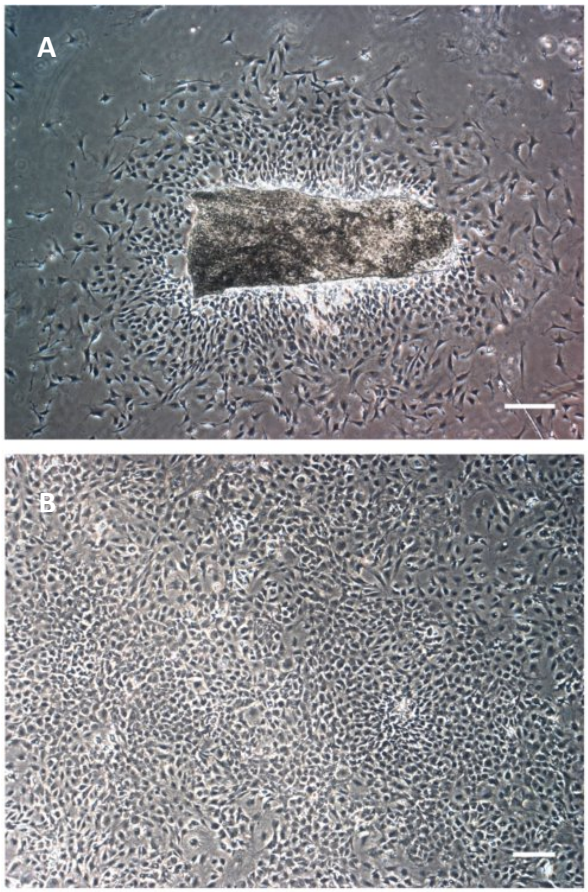


Figure 37. Isolation of osteoblasts from calvaria of 7-day-old oim mice.

Oim osteoblasts isolated by A) culturing bone chips explants and B) trypsin-collagenase digestion, after 6 days in culture with αMEM. Scale bar: 200 µm.

Oim osteoblasts were cultured for two weeks in osteogenic medium supplemented with conditioned medium derived from human second trimester AFSCs (n=3) in a 1:1 ratio. At the end of the experiment, *oim* osteoblasts were collected and RNA was extracted, to assess the expression of osteogenic markers. *Oim* osteoblasts cultured in osteogenic medium, supplemented with proliferative medium in a 1:1 ratio, were used as control (n=3). The gene expression ($2^{-\Delta\Delta Ct}$) of *Runx2*, *Alp1* (alkaline phosphatase) and *Bglap* (osteocalcin) was dramatically decreased in *oim* osteoblasts cultured with AFSC-conditioned medium, compared to controls (**Figure 38**). This reduction was possibly due to the exhaustion of the conditioned medium by human AFSCs.

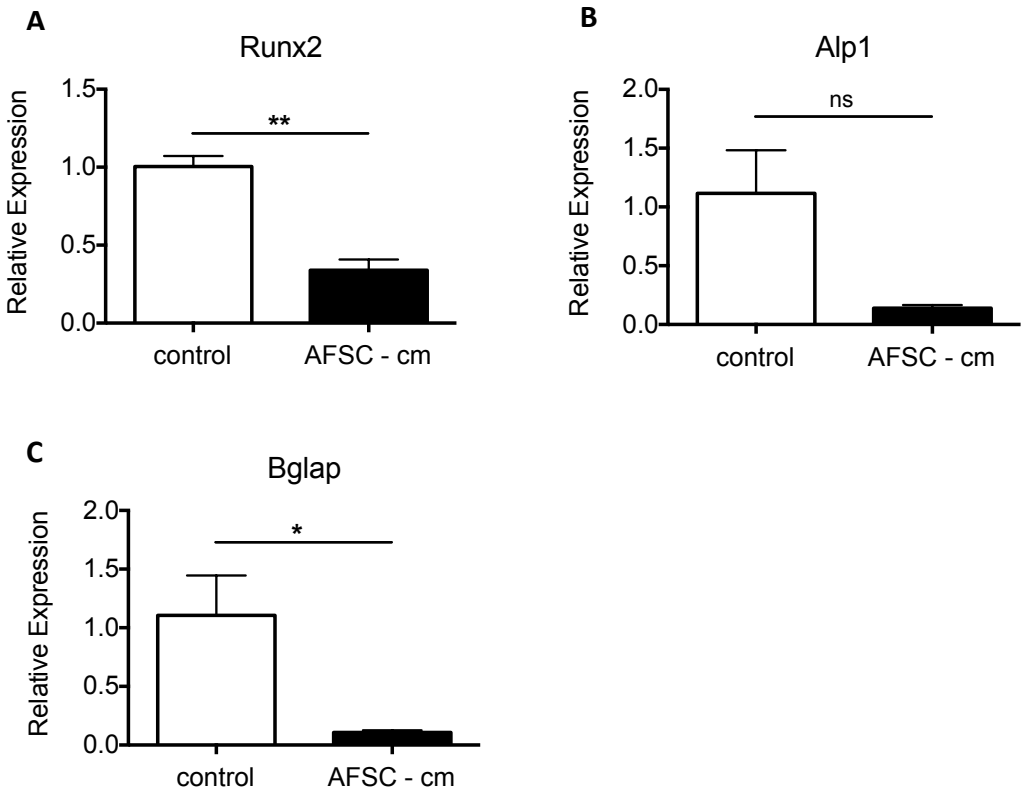


Figure 38. AFSC-conditioned medium decreases the expression of osteogenic markers in oim osteoblasts in vitro.

*Quantitative real time PCR of expression of A) Runx2, B) Alp1 and C) Bglap in oim osteoblasts cultured for 2 weeks in osteogenic αMEM supplemented with human AFSC-conditioned medium (50%) (n=3), compared to oim osteoblasts cultured in non-supplemented osteogenic medium (n=3). Data represent mean ± SEM * P<0.05 and ** P<0.01 (Student's t-test).*

6.2.2 Co-culture of human AFSCs and MG-63 osteoblast-like cells

Due to the difficulty in maintaining the ability of *oim* osteoblasts to differentiate after repeated passaging, because of senescence, other cell sources were identified to further investigate the effect of AFSCs on osteoblasts. The MG-63 human osteosarcoma cell line was used as cell source to model the effect of AFSCs in a 'human-on-human' system. MG-63 cells are osteoblast-like cells, easy to grow without reaching senescence, however they are not as physiologically relevant as primary cells and exhibit a heterogeneous expression profile of osteogenic markers (Pautke et al., 2004). MG-63 cells were cultured for two weeks in osteogenic medium supplemented with concentrated (x200) conditioned medium derived from human second trimester AFSCs (n=3). MG-63 cells cultured in osteogenic medium were used as controls. At the end of the experiment, cells were collected to extract RNA and assess the expression of osteogenic markers. The gene expression levels ($2^{-\Delta\Delta Ct}$) of the osteogenic markers osteopontin (*Opn*) and osteocalcin (*Bglap*) were very low and not significantly affected by the AFSC-conditioned medium (**Figure 39**).

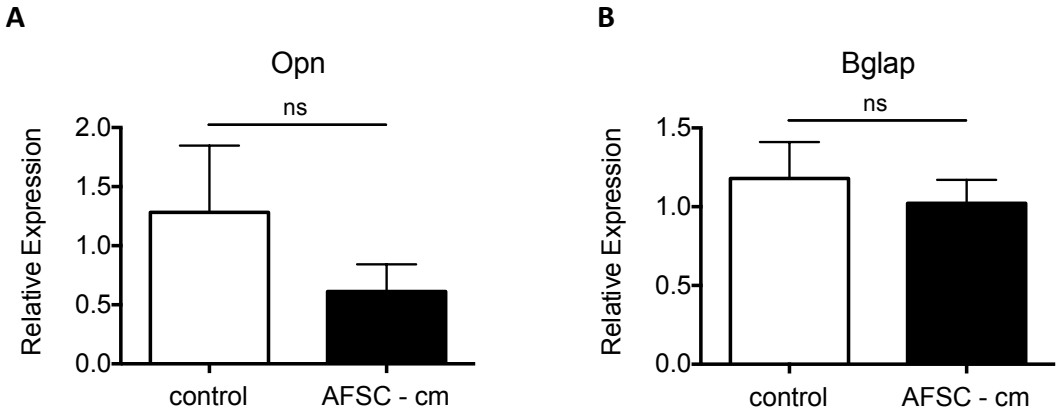


Figure 39. Concentrated AFSC-conditioned medium does not affect the expression of osteogenic markers in MG-63 osteoblast-like cells.

Quantitative real time PCR of expression of A) Osteopontin and B) Bglap in MG-63 cells cultured for 2 weeks in osteogenic αMEM supplemented with concentrated (x200) human AFSC-conditioned medium (n=3), compared to MG-63 cells cultured in non-supplemented osteogenic medium (n=3). Data represent mean ± SEM (Student's t-test).

6.2.3 Optimisation of a protocol for the isolation of human fetal osteoblasts from calvarial tissue

In order to achieve a 'human-on-human' in vitro model to further investigate the effect of AFSCs on osteoblasts, a more physiologically relevant cell source was necessary. Calvarial bones were obtained from fetal samples derived from voluntary terminations of pregnancy, obtained through a collaboration with the MRC-Wellcome Trust Human Developmental Biology Resource (HDBR), based at the UCL GOS Institute of Child Health. The isolation protocol for osteoblasts used for rodent tissues, developed by (Orriss et al., 2012; Taylor et al., 2014) and consisting of sequential digestions with 0.25% trypsin-EDTA and 0.2% collagenase type II in Hanks balanced salt solution (HBSS), was adapted and optimised for human fetal tissue. A detailed protocol can be found in the "Materials and methods" chapter. This work was performed with Dr Rachel Sagar.

Calvarial tissue was dissected, cut in small pieces and subsequently washed thoroughly in PBS. In order to optimise the protocol, fragments were then treated with different 15-minute trypsin-EDTA incubation cycles, followed by 2 cycles of 1-hour collagenase incubation. For every cycle, the solution was changed with fresh trypsin-EDTA. The highest number of cells retrieved was observed after 1 cycle of trypsin-EDTA, although there was no statistical difference in the number of cells obtained after 1 to 4 cycles. Cells obtained were $18.2 \times 10^4 \pm 1.8 \times 10^4$ after 1 cycle, $16.0 \times 10^4 \pm 1.9 \times 10^4$ after 2 cycles, $9.6 \times 10^4 \pm 3.6 \times 10^4$ after 3 cycles and $13.2 \times 10^4 \pm 1.2 \times 10^4$ after 4 cycles of trypsin-EDTA (**Figure 40A**).

One cycle of trypsin-EDTA was selected and used to then optimise the collagenase protocol. Bone fragments were incubated with collagenase solution for 30 minutes, 1 hour and 2 cycles of 1 hour each. The highest number of cells were isolated after 1 hour of digestion, however the number of cells did not significantly differ in the three conditions. Cells obtained were $7.9 \times 10^4 \pm 0.3 \times 10^4$ after 30 minutes of collagenase incubation, $15.5 \times 10^4 \pm 3.6 \times 10^4$ after 1 hour and $9 \times 10^4 \pm 2.4 \times 10^4$ after 2 x 1-hour cycles of collagenase digestion (**Figure 40B**). A protocol consisting of 15 minutes of

0.25% trypsin-EDTA followed by 1 hour of 0.2% collagenase type II was selected for the isolation of human fetal osteoblasts from the calvaria.

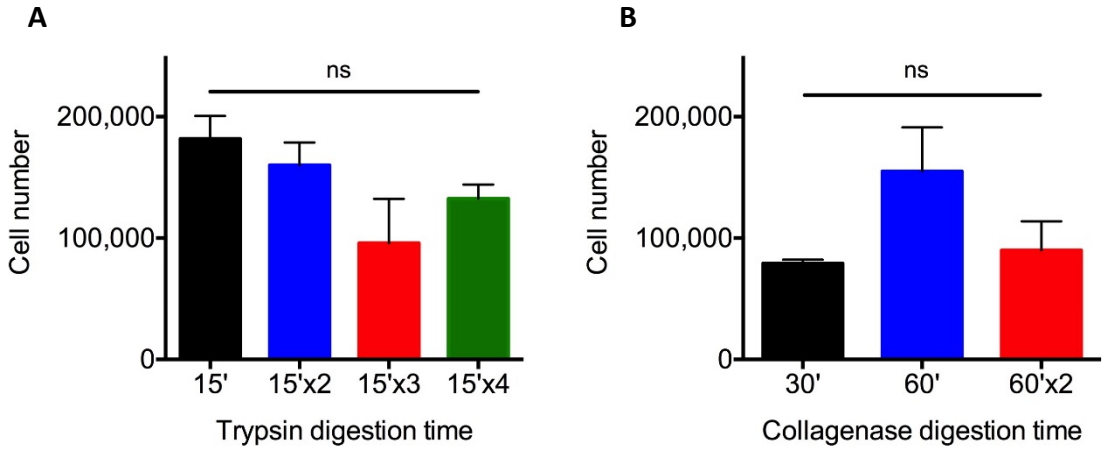


Figure 40. Optimisation of a protocol for the isolation of osteoblasts from human fetal calvaria.

Comparison of number of cells isolated from human fetal calvaria (20 pcw) by A) different trypsin-EDTA and B) collagenase type II digestion times. Data represent mean ± SEM (One-way ANOVA followed by Bonferroni post hoc test).

Following the optimisation of the isolation protocol, different culture conditions were tested with the aim of finding the ideal osteogenic differentiation protocol for calvaria-derived human fetal osteoblasts. The concentration of β-glycerophosphate has been identified as critically important in the osteogenic differentiation of osteoblasts. The absence of β-glycerophosphate prevents mineralisation, while excessive concentrations cause non-selective widespread deposition of minerals, thus impairing cell viability (Orriss et al., 2012). Cells at passage 3 were seeded at high density (20,000 cells/cm^2) and cultured in DMEM or αMEM supplemented with 0.2 mM ascorbic acid and 10 nM dexamethasone and 2.5 or 5 mM β-glycerophosphate. Human fetal osteoblasts formed dense regularly structured networks after 2 weeks when cultured in osteogenic αMEM with 5 mM β-glycerophosphate, to a lesser extent in the 2.5 mM condition, and they formed only sparse structures when cultured in the DMEM conditions (**Figure 41A**). Deposition of calcium minerals was confirmed by alizarin red staining (**Figure 41B**). Bone formation was assessed by

quantifying the percentage of mineralised area. When cultured in DMEM, human fetal osteoblast bone network covered 10.7 % ± 1.6 in the 2.5 mM and 19.1 % ± 1.0 in the 5mM β-glycerophosphate condition. When cultured in αMEM, the area covered was 38.9 % ± 0.2 in the 2.5 mM and 56.4 % ± 2.0 in the 5mM β-glycerophosphate condition (**Figure 41C**).

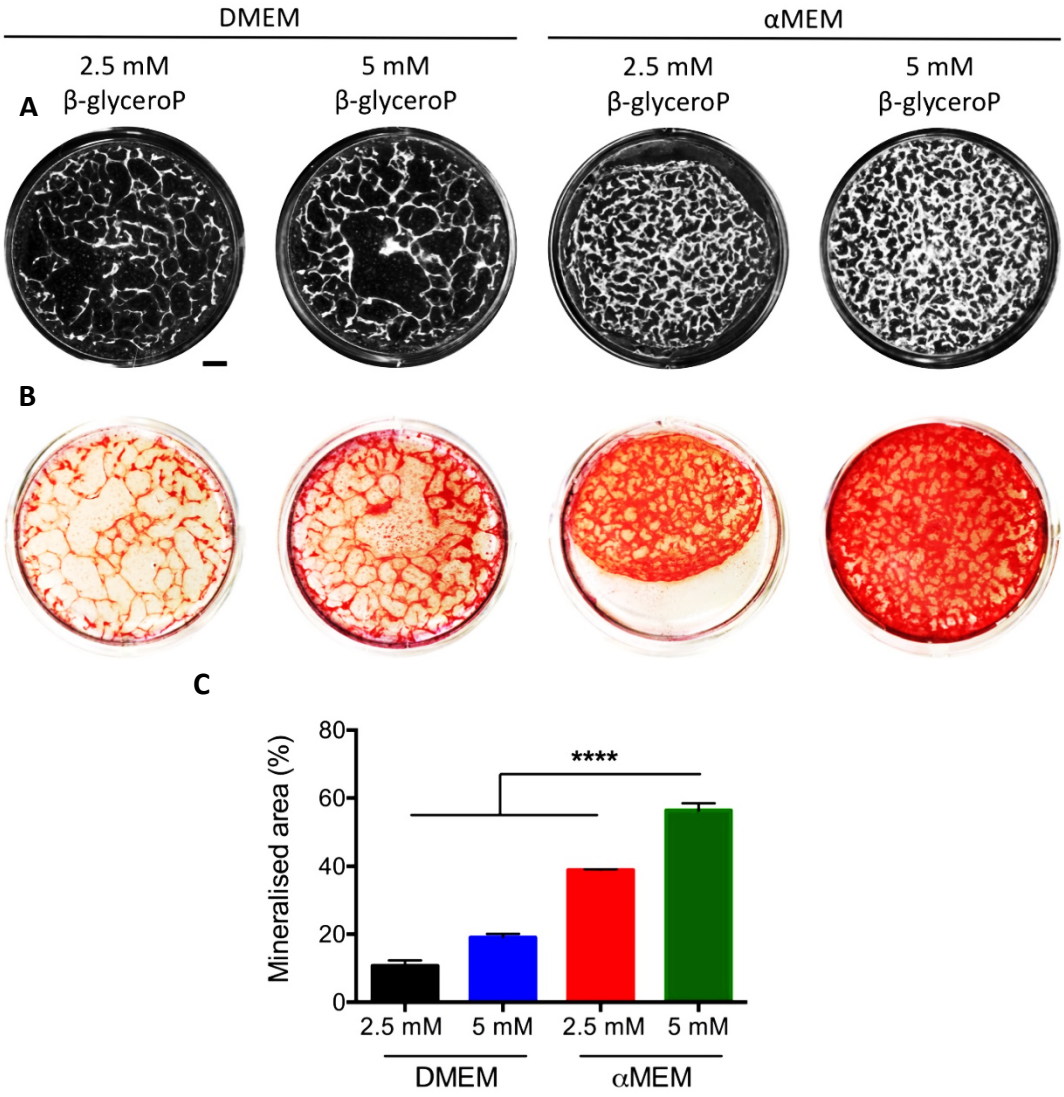


Figure 41. Optimisation of culture conditions for the osteogenic differentiation of human fetal osteoblasts.

*A) Bone trabecular networks formed by human fetal osteoblasts (20 weeks post conception) derived from the calvaria, cultured in different osteogenic conditions for 2 weeks. Scale bar: 2 mm. B) Alizarin red staining confirming calcium deposition. C) Quantification of the area covered by mineralised bone. Data represent mean ± SEM **** P<0.0001 (One-way ANOVA followed by Bonferroni post hoc test).*

Calvaria-derived human fetal osteoblasts, from different gestational ages, cultured in αMEM supplemented with 0.2 mM ascorbic acid, 10nM dexamethasone and 5mM β-glycerophosphate showed dense trabecular bone network formation and magnified visualisation through a phase contrast microscope revealed selective mineralisation, which left the underlying osteoblast cell layer intact and viable (**Figure 42**).

Two photon lifetime imaging (FLIM) analyses were used to confirm the deposition of collagen by human fetal osteoblasts cultured in osteogenic medium. Second harmonic signal was detected (**Figure 43**) and two lifetimes of fluorescence decay of the autofluorescence were measured: 0.34(+/-0.01) ns and 2.69(+/-0.02) ns. Given that the longer lifetime was obtained by 26.6(+/-0.3) % of the signal, the resulting mean lifetime was 0.97(+/-0.02) ns. These data are in agreement with those corresponding to collagen type I (Shirshin et al., 2017), thus confirming the deposition of collagen by human fetal osteoblasts.

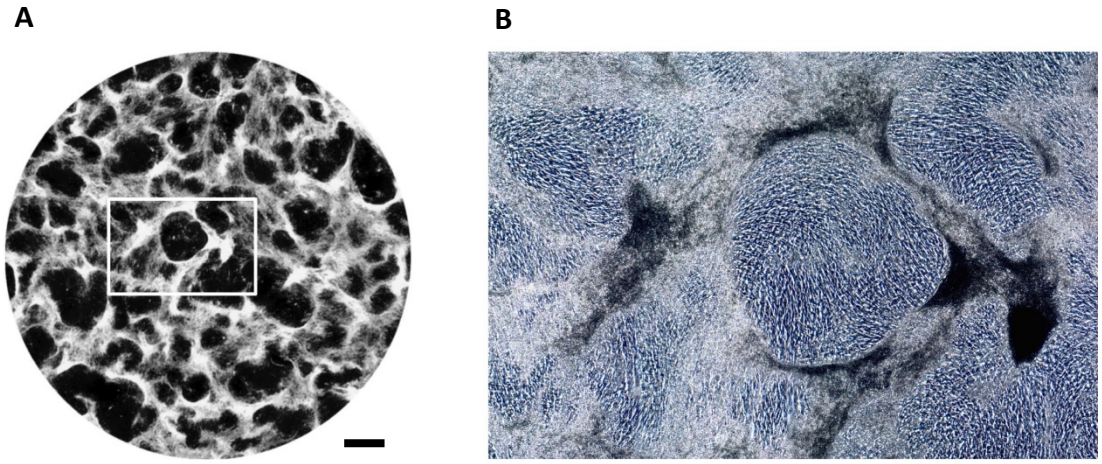


Figure 42. Human fetal osteoblasts form trabecular bone networks in vitro.

A) Bone trabecular networks formed by human fetal osteoblasts (13 weeks post conception) derived from the calvaria. Unstained osteoblast culture imaged by reflected light scanner. B) Phase contrast magnification of trabecular network showing selective mineralisation. Scale bar: 2 mm.

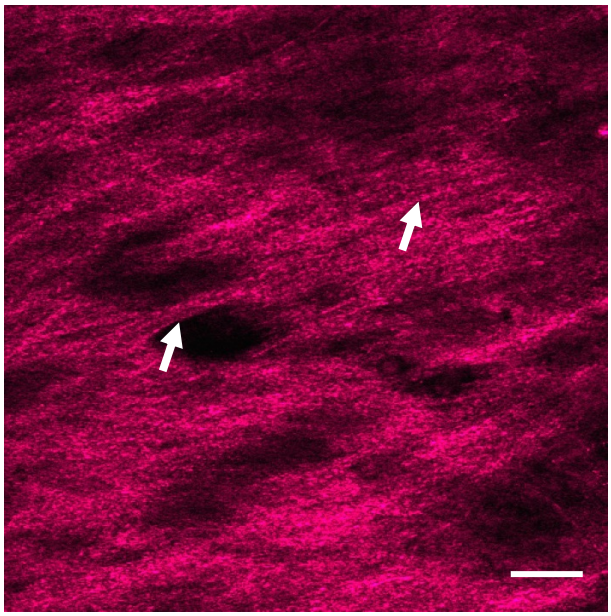


Figure 43. Collagen deposited by human fetal osteoblasts visualised by dual-photon microscopy.

Second harmonic generation signal showing collagen type I fibres (arrows) deposited by human fetal osteoblasts derived from calvarial tissue (13 weeks post-conception) and cultured for 2 weeks in osteogenic medium. Scale bar: 20 µm. In collaboration with Dr Thomas Blacker and Prof Michael Duchen, UCL Department of Cell and Developmental Biology.

Human calvaria-derived fetal osteoblasts showed a high expansion potential and were able to grow after repeated passages. In order to assess the proliferation rate of cells isolated from different gestational ages, osteoblasts derived from samples of 9, 12 and 16 weeks post-conception (wpc) (n=1 per gestational age) were cultured and their growth charted for 5 passages. The growth rate was assessed by seeding cells at low density (5,000 cells/cm^2) and charting their growth over a period of 18 days, throughout which the cell number was counted and the same number of cells re-seeded every 3 days. Interestingly, cells from older gestational age grew faster than cells from younger donors. Specifically, osteoblasts from the 16 wpc sample reached 7.3 ± 0.7 population doublings in 18 days, compared to cells from the 12 wpc sample which reached 6.7 ± 0.3 population doublings and cells from the 9 wpc sample that achieved 4.4 ± 0.2 ($P<0.01$) However, the cells from 16 weeks sample appeared to stop growing around day 15. (**Figure 44**).

Thanks to the samples and the karyotype data kindly provided by the MRC-Wellcome Trust Human Developmental Biology Resource (HDBR), a biobank consisting of 24 human fetal osteoblast lines isolated from the calvaria was built, with sample gestational ages ranging from 9 to 21 weeks post-conception (**Table 7**).

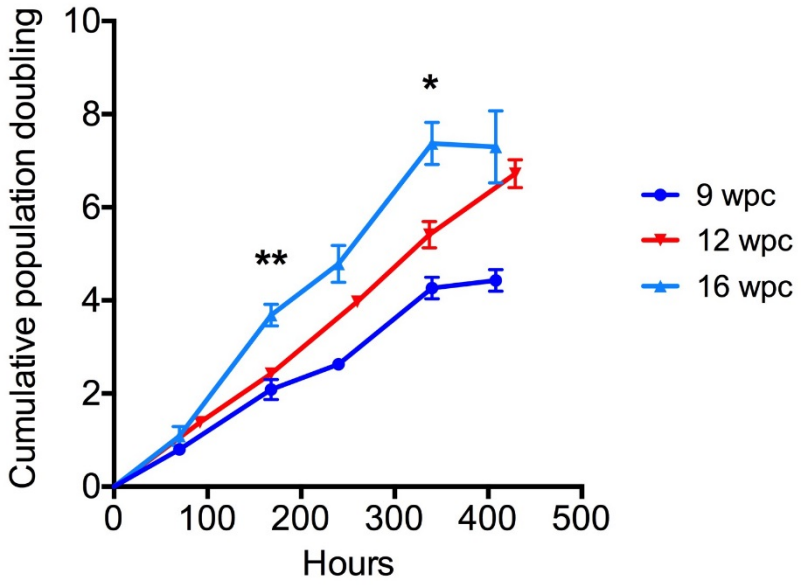


Figure 44. Human fetal osteoblasts from older gestational ages grow faster than younger ones.

*Cumulative population doublings of human fetal osteoblasts isolated from calvaria of 9, 12 and 16 weeks post conception (wpc), measured for a period of 18 days (n=1 for each gestational age, data collected in technical replicates). Data represent mean ±SEM ** P<0.01 (One-way ANOVA followed by Bonferroni post-hoc test).*

Sample number	Tissue	Gestational age	Karyotype
1	Calvaria	9 wpc	46 XX
2	Calvaria	10 wpc	46 XY
3	Calvaria	11 wpc	46 XX
4	Calvaria	12 wpc	46 XX
5	Calvaria	12 wpc	46 XY
6	Calvaria	13 wpc	46 XX
7	Calvaria	13 wpc	46 XY
8	Calvaria	13 wpc	46 XY
9	Calvaria	13 wpc	46 XX
10	Calvaria	13 wpc	46 XY
11	Calvaria	15 wpc	46 XY
12	Calvaria	15 wpc	46 XY
13	Calvaria	15 wpc	46 XX
14	Calvaria	16 wpc	46 XY
15	Calvaria	17 wpc	46 XX
16	Calvaria	17 wpc	46 XX
17	Calvaria	19 wpc	46 XX
18	Calvaria	19 wpc	46 XY
19	Calvaria	20 wpc	46 XX
20	Calvaria	20 wpc	46 XY
21	Calvaria	20 wpc	46 XY
22	Calvaria	20 wpc	46 XY
23	Calvaria	21 wpc	46 XY
24	Calvaria	21 wpc	46 XX

Table 7. Human fetal osteoblast biobank.

Human fetal calvarial osteoblasts were isolated from samples of different gestational ages, ranging from 9 to 21 weeks post-conception (wpc).

6.2.4 Co-culture of human AFSCs and human fetal osteoblasts

The human fetal osteoblasts isolated and banked using the protocol described above were then used in a preliminary investigation of the effects of AFSC-conditioned medium on osteoblasts, in a 'human-on-human' system.

Human fetal osteoblasts from 13 weeks post conception samples were cultured for two weeks in osteogenic medium supplemented with concentrated (x200) conditioned medium derived from either naïve or TGF-β- and TNFα- stimulated AFSCs (5 ng/ml, 24-hour stimulation). Human fetal osteoblasts cultured in osteogenic medium were used as controls. At the end of the experiment, cells were collected to extract RNA and assess the expression of osteogenic markers. Interestingly, the level of expression of osteopontin (*Opn*) was significantly reduced after exposure to naïve AFSC-conditioned medium, while no changes were seen when AFSCs were stimulated with TGF-β and TNFα (**Figure 45A**). Osteocalcin (*Bglap*), did not show any changes in its expression, which remained very low in all the conditions (**Figure 45B**).

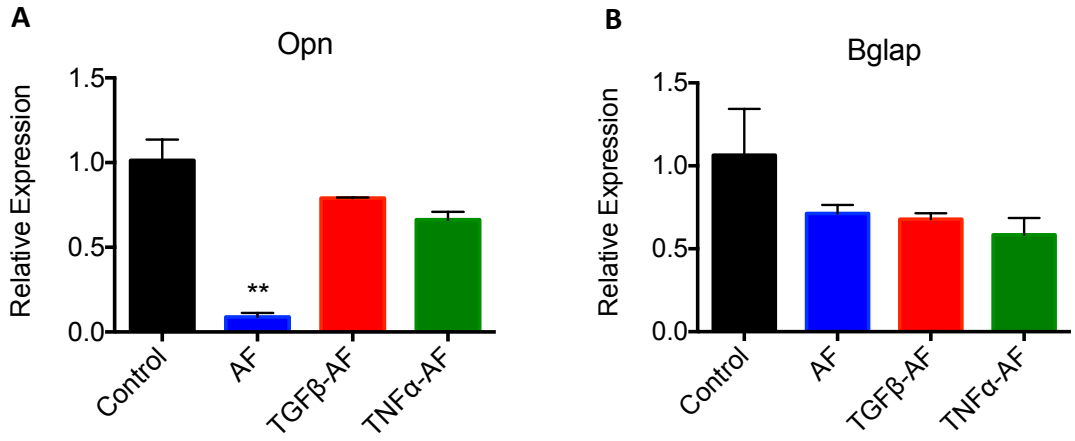


Figure 45. Concentrated AFSC-conditioned medium does not affect the expression of osteogenic markers in human fetal osteblasts.

*Quantitative real time PCR of expression of A) Osteopontin and B) Bglap (osteocalcin) in human fetal osteoblasts (13 wpc) cultured for 2 weeks in osteogenic αMEM supplemented with concentrated (x200) medium conditioned by naïve AFSCs, TGF- β–stimulated AFSCs and TNFα-stimulated AFSCs, compared to human fetal osteoblasts cultured in non-supplemented osteogenic αMEM. Data represent mean ± SEM ** P<0.01 (One-way ANOVA followed by Bonferroni post hoc test).*

6.3 Discussion

These data show that exposing osteoblasts to AFSC-conditioned medium did not mimic the therapeutic effects of *in vivo* stem cell administration in the *oim* model. Moreover, when osteogenic medium was supplemented with conditioned medium from naïve AFSCs, the expression of osteopontin in human fetal osteoblasts decreased significantly.

Many reasons could explain this: conditioned medium is not an optimal experimental setting, since medium can be exhausted from the conditioning cell type. Concentrating conditioned medium and supplementing it to fresh medium is useful to avoid exhaustion, however the concentration step is carried out by centrifuging medium through filters. Although the smallest pores size was selected to avoid losing small cytokines, the filters used had a cut-off of 3 kDa, which cannot ensure the retention of small factors. Moreover, optimal *in vitro* osteogenic differentiation protocols involve long-term culture, ranging from 4 weeks for rodent cells to 2 weeks for human fetal osteoblasts (Orriss et al., 2012). Long-term culture without passaging can cause cell apoptosis and senescence and represents a major risk of contamination. More work with different experimental approaches is therefore needed to elucidate the paracrine effect of stem cells on osteoblasts in the *oim* model.

Different mechanisms of actions are most certainly involved and need to be investigated to explain the therapeutic effects described in the previous chapters, despite the low engraftment of donor cells found. Such mechanisms, that are further discussed in the general discussion, include direct contact between AFSCs and target resident cells, the effect of chimeric extracellular matrix formed by collagen produced in part by AFSCs and the immunomodulatory properties of MSCs.

The isolation and *in vitro* differentiation of osteoblasts has been crucial for the study of bone development and for the understanding of human bone pathologies. Most *in vitro* studies have been performed with murine osteoblasts, which are easy to isolate and maintain but have limited proliferation and differentiation potential and may

behave differently than human cells. Human fetal osteoblasts are better alternatives to those isolated from adult biopsies, since they have high proliferation and differentiation potential (Gothard et al., 2015). The protocol described in this chapter is simple and reproducible and yields a high number of osteoblasts, which can be useful for the modelling of human bone pathologies and as a platform for drug testing.

Chapter 7

General discussion, conclusions and future work

7.1 Background

The work presented in this thesis builds on previous studies investigating of the potential of cell therapy for OI. Since the first stem cell transplantation in a mouse model of OI, in 1998, more than ten preclinical and clinical studies have been published, each representing a step forward towards the translation of stem cell therapy for OI in clinics (Pereira et al., 1998).

The first attempts involved the transplantation of murine cells in mouse models of OI and focused on proving the feasibility of transplantation, avoiding immune rejection and achieving engraftment of donor cells in recipient target tissues (Pereira et al., 1998; Wang et al., 2006; Li et al., 2007). Those studies paved the way for further investigation in a human on human system. At the same time, progress in basic research led to the discovery of the immune-privileged properties of MSCs, which allow transplanted cells to escape being recognised by host immune cells (Liechty et al., 2000). Human stem cells began to be used in preclinical studies of cell therapy for OI and the focus became the identification of the most suitable route of administration. In preclinical studies different ways were investigated, including intra-peritoneal (Jones et al., 2012a; 2014), intra-venous, *in utero* (Guillot et al., 2008a; Panaroni et al., 2009; Vanleene et al., 2011) and local administration in bones (Li et al., 2010a; Pauley et al., 2014). OI is a collagen type I disorder and it therefore involves the entire body, including the skeletal system. Systemic administration route, despite achieving lower levels of engraftment in single bones than the intra-femur injection and causing off-target homing of cells, which especially remain trapped in the lungs, allows better distribution of transplanted cells and appears therefore to be the best option for cell therapy in OI.

In the 20 years since the first transplantation of MSCs in children with severe OI, only 10 patients have received cell therapy for OI, both before and after birth. Remarkable improvements of disease phenotype were observed, although limited in the 6-12 months after transplantation, thus requiring multiple infusions (Horwitz et al., 1999; 2002; Le Blanc et al., 2005; Götherström et al., 2014).

Clinical translation of cell therapy for OI is now closer than ever, with the first registered clinical trial for pre- and post-natal transplantation of allogeneic fetal liver MSCs in children with OI underway across Europe at the moment. Results from the trial will provide important information on the long-term safety and efficacy of stem cell therapy, including the optimal dose and whether it would be better to transplant before or after birth (Chitty et al., 2016).

Despite these impressive scientific, surgical and technological advances, many challenges remain to be addressed before stem cell transplantation for OI can be routinely implemented in clinics.

7.2 Advantages of using AFSCs for cell therapy

One of the key issues remains finding the most relevant stem cell type to use. The optimal source for cell therapy for OI needs to be accessible without ethical restrictions and easy to isolate in large numbers. Moreover, cells need to have a high proliferative potential, to be able to sustain extensive expansion *in vitro*, in the event of repeated transplantations, and to facilitate cell survival and proliferation in vivo following transplantation. Finally, the optimal cell source needs to meet safety criteria, including not being susceptible to attack by the immune system of the host and not being able to form tumours after transplantation.

Fetal MSCs, including those derived from the liver and used clinically in the first *in utero* transplantation in children with severe OI, meet all these criteria (Götherström, 2016). However, many drawbacks limit the use of fetal liver MSCs: they are obtained from terminated pregnancies, are difficult to isolate and are available in small numbers, requiring long-term expansion in culture.

In recent years, more ethical sources of fetal stem cells that meet these criteria have been investigated for their potential in the field of regenerative medicine, including extraembryonic tissues such as the placenta, the umbilical cord and the amniotic fluid (De Coppi et al., 2007; Soncini et al., 2007; Joerger-Messerli et al., 2016; Antoniadou

and David, 2016). Human fetal chorionic cells, obtained by either diagnostic chorionic sampling or from discarded placentae at birth, have been investigated by Dr. Guillot's lab as a valid alternative for cell therapy in the *oim* model (Jones et al., 2014a).

This thesis proposes AFSCs as a cell source for transplantation for the treatment of OI. AFSCs have numerous advantageous characteristics that make them promising candidates for cell therapy. AFSCs are available throughout pregnancy and can be safely isolated during second trimester routine amniocentesis, typically between the 14^{th} and the 16^{th} week of gestation, during third trimester amnioreduction in the case of excessive amniotic fluid or at the end of gestation, during caesarean section. The amniotic fluid is therefore a highly accessible source of stem cells, not limited by the ethical and legal restrictions that restrain the use of other cell types with potential for regenerative medicine, such as embryonic stem cells or fetal cells that rely on terminated pregnancies (Loukogeorgakis and De Coppi, 2016). While other extra-embryonic tissues such as the umbilical cord and the placenta are rich sources of stem cells available at birth, AFSCs can be safely isolated also during pregnancy, which could allow autologous use for perinatal therapy (Loukogeorgakis and De Coppi, 2017).

Other characteristics of AFSCs useful for regenerative medicine purposes are their high proliferative potential and broad differentiation capacity, which are however not accompanied by the ability to form teratomas *in vivo*. It has become clear that AFSCs show characteristics that are consistent with a broadly multipotent phenotype. Other than the three mesodermal lineages, AFSCs are in fact able to differentiate *in vitro* into cells of endothelial, hepatic and neuronal lineages (Roubelakis et al., 2007). In contrast to their primitive phenotype, when transplanted in immunocompromised mice, AFSCs are unable to form tumours (Moschidou et al., 2012; Loukogeorgakis and De Coppi, 2017).

Due to this advantageous trade-off between proliferation and differentiation potential and safety, AFSCs have been investigated in preclinical studies for the treatment of a number of pathologies. For example, intravascular injection of rat allogeneic AFSCs has been reported to ameliorate heart failure and decrease

inflammation in a rat model of pulmonary hypertension (Castellani et al., 2013). Therapeutic effects were also obtained following intraperitoneal administration of AFSCs in a rat model of necrotizing enterocolitis and in a mouse model of muscular dystrophy (Zani et al., 2014; Loukogeorgakis and De Coppi, 2017).

7.3 AFSCs are promising candidates for the treatment of OI

The study carried out for this thesis represents the first investigation of the therapeutic effects of human amniotic fluid stem cells in a mouse model of OI.

Transplanted AFSCs migrated preferentially to the *oim* mice bones where they were detected, through estimating the levels of human mRNA, eight weeks after injection. Histological analyses showed donor cells localised preferentially at sites of active bone formation and differentiated *in vivo* into functional osteoblasts, able to produce collagen type I and osteopontin. The relatively low level of engraftment and differentiation was in line with previous studies which used both murine and human MSCs, that estimated 2-10% engraftment in the *oim* model. The ability of donor AFSCs to contribute to the ECM formation through production of the Col1a2 chain, which is missing in the *oim* model, are consistent with data from other groups using different types of MSCs, supporting MSCs as a suitable source of cells for bone regenerative medicine of bone disorders (Li et al., 2007; Guillot et al., 2008a; Panaroni et al., 2009; Vanleene et al., 2011; Jones et al., 2014).

AFSC transplantation led to a 79% decrease in the number of fractures, compared to non-transplanted *oim* mice. The ability of human fetal MSCs to reduce fractures in the long bones has been reported previously by other studies. Reductions of fracture numbers, following transplantation with human fetal blood and placental MSCs, ranged between 66% to 84%, which is in line with the findings of this study (Guillot et al., 2008a; Vanleene et al., 2011; Jones et al., 2014).

In the *oim* model, bone fragility is caused by the simultaneous reduction of bone quantity and quality. In order to assess the therapeutic effects of transplantation,

analyses of the biomechanical properties of bones and of their structural organisation are therefore essential (Chipman et al., 1993; Shapiro et al., 2009).

Three-point bending analyses showed that transplantation of AFSCs significantly increased the biomechanical qualities of *oim* femurs. In particular the stiffness of bones and the work needed to reach the breaking point, which are dramatically reduced in *oim* femurs compared to wild type, were significantly increased after AFSC transplantation. These data demonstrate that transplantation of AFSCs increases the strength of *oim* bones. In previous studies using other sources of fetal MSCs, transplantation failed to impact long bone strength and stiffness, despite reducing fracture rate. Transplantation of fetal blood MSCs led to an 84% reduction of fracture numbers, but was not concomitant with changes in the biomechanical properties measured in femurs, which remained the same as non-transplanted *oim* bones (Vanleene et al., 2011). Conversely, transplantation with placenta-derived chorionic MSCs, which reduced the overall number of long-bone fractures by 66%, caused a significant decrease in bone stiffness. The fewer fractures observed following transplantation were explained by the increased ductility of bones, possibly because of the production of normal collagen that normalises the ECM. This theory was supported also by the increase of the maximum deflection before fracture achieved in transplanted bones compared to *oim* bones. Moreover, bone strength remained unchanged (Jones et al., 2014).

In this study, AFSC engraftment levels were positively correlated with increase in bone strength and stiffness, suggesting that, at least in part, donor cells directly contributed to the improvement of biomechanical properties. Interestingly, placental stem cell engraftment level was inversely correlated with bone stiffness, supporting the theory that placental cells may ameliorate bone quality by impacting on bone ductility rather than stiffness (Jones et al., 2014).

Micro-structural analyses by micro-CT showed that AFSC transplantation did not have any effects on cortical or trabecular bone volume, trabecular number and thickness and on total porosity. The only parameter that was significantly improved following transplantation was the level of connectivity of the trabeculae, which was estimated

by measuring the trabecular pattern factor (Hahn et al., 1992). In this study, the increase of bone strength was therefore attributed to the structural organisation of trabecular bone. In the case of fetal blood MSC transplantation, bone volume and trabecular parameters analysed by micro-CT remained also unchanged despite transplantation and the connectivity of trabeculae was not assessed. On the other hand, placental cell administration significantly increased tibial bone volume, while other trabecular and cortical parameters analysed remain similar to those measured in non-transplanted *oim* bones (Jones et al., 2014).

Taken together, the data presented in this thesis and the studies carried out by other authors suggest that distinct cell sources, although similar in their phenotype and characteristics, as is the case of placental and amniotic fluid MSCs, may improve the quality of *oim* bones through very different mechanisms.

The disease phenotype of OI is in part caused by the impaired maturation of osteoblasts, which are unable to fully differentiate, thus leading to increased osteoclastogenesis and bone turnover that is shifted towards bone resorption (Kalajzic et al., 2002; Martin et al., 2011; Gioia et al., 2012). Recently, osteoblast differentiation impairment has been linked to the formation of a disorganised ECM, which fails to bind proteoglycans such as decorin that are responsible for the sequestration of excessive TGF-β (Grafe et al., 2014b). TGF-β is a strong inhibitor of osteoblast and an stimulator of osteoclast activity when present at excessive levels (Hattersley and Chambers, 1991; Alliston et al., 2001).

Transplantation of AFSCs appears to improve the differentiation of *oim* osteoblasts, as shown by the higher expression of genes involved in mouse skeletal development, ECM formation and osteoblast activity. These data appear to support the theory that increased osteoblast differentiation leads to normalisation of bone remodelling, as shown by the decrease in RANKL expression. Gene array data are however not conclusive as they only derived from two *oim* and two transplanted samples. In a similar trend, the aforementioned study using placental stem cells showed an increase in the expression of genes involved in endochondral and intramembranous ossification. In particular, genes involved in hypertrophic chondrocytes

differentiation were upregulated following placental chorionic MSC transplantation (Jones et al., 2014).

7.4 Mechanism of action

In the context of developing a stem cells therapy for clinical translation, the understanding of the mechanism of action of donor cells and of their fate following transplantation is of major importance. Different mechanisms of action can be speculated for the explanation of the therapeutic effects observed in the *oim* model following transplantation of AFSCs.

Engraftment data from this thesis show that AFSCs migrated towards the bones, homed there and differentiated into osteoblasts *in vivo*. Moreover, the improvement of bone mechanical properties such as stiffness and total work to fracture positively correlated with the number of cells engrafted. These data support the classic theory of cell therapy, which postulates that donor cells exert anabolic functions by differentiating and replacing defective cells, thus directly contributing to new "healthy" bone formation by participating in the generation of chimeric ECM (Spees et al., 2016). This theory builds on studies investigating the phenomenon of naturally occurring mosaicism in OI families. OI mosaic carriers show little or no symptoms despite having a high proportion of mutated cells, ranging from 40% to 75% of the total number of osteoblasts (Dalgleish, 1998; Cabral and Marini, 2004) . Those data suggested that defective collagen can be incorporated at a high percentage in the ECM without causing major impairment to bone quality and strength and that there might be a threshold level of mutant collagen that needs to be reached in order to exhibit any OI skeletal symptoms. It is therefore possible that a small number of normal cells, able to produce the missing collagen chain, would be enough to normalise the quality of the defective matrix observed in the *oim* model (Gerson, 1999).

However, the percentage of engraftment reported by this as well as other studies in the *oim* model does not appear to reach significant levels and durations sufficient to

entirely justify the therapeutic effects seen in terms of tissue regeneration. The donor-resident chimerism levels of the matrix described in these studies seem unlikely to be enough to directly sustain the improvements in bone quality and quantity described by these studies. It appears more likely that they are a consequence of the migratory and plasticity properties of human fetal MSCs, which are able to undergo efficient osteogenic differentiation when homed to a permissive environment. This is in line with a growing trend in regenerative medicine that postulates that other mechanisms might take part and need to be considered to explain the therapeutic effects observed in cell therapy studies, despite low level of cell survival and proliferation (Spees et al., 2016).

Recently, the interest of the stem cell community has shifted towards the investigation of the paracrine effects that MSCs exert after being transplanted in a damaged or inflamed tissue. This is exemplified by the developmental origin and physiological functions of MSCs, which act as stromal support cells to sustain the hematopoietic and endothelial niches during development (Sacchetti et al., 2007; Crisan et al., 2008). Transcriptome and proteome analyses have demonstrated that MSCs produce a wide variety of trophic factors, including anti-inflammatory and immune-modulatory cytokines and growth factors, known for promoting cell survival and having angiogenetic properties (Silva et al., 2003; Caplan and Dennis, 2006; Phinney et al., 2006). It has been reported that conditioned medium derived from MSC cultures can act as replacement to cell product administration in preclinical studies for heart infarction and acute kidney injury (Bi et al., 2007; Iso et al., 2007; Spees et al., 2016).

An alternative hypothesis to explain the findings presented in this thesis is therefore that the anabolic therapeutic effects of AFSC transplantation were mediated by the release of growth factors that promoted the osteogenic differentiation of the defective *oim* pre-osteoblasts, rather than by the direct differentiation of AFSCs. This theory is supported by the gene array data that show a general upregulation of endogenous genes involved in the skeletal development and resident osteoblast differentiation. AFSCs are known to produce anti-inflammatory cytokines as well, particularly in response to an inflammatory environment such as the one observed in

OI due to the damaged tissues and bone fractures. It is therefore possible that the therapeutic effects observed are due to the dampening of the inflammation following transplantation. The gene expression analyses of two key inflammatory factors TNFα and NFKB, however, did not show any decrease in the inflammatory status of AFSC-transplanted *oim* compared to non-transplanted mice, suggesting that this pathway might not be relevant in this case. It is however important to note that these two genes are also known for having other functions and they might not be the most relevant markers to assess tissue inflammatory status. Also priming AFSCs with inflammatory factors such as TGF-β and TNFα did not lead to any changes in the maturation of osteoblasts that were cultured in conditioned medium derived from primed-AFSCs cultures. However, these observations were limited to few genes and more studies focused on investigating the influence that AFSC-derived products have on the inflammatory status in OI are needed to better understand the mechanism of action of AFSC-based cell therapies.

A third possible explanation builds on recent work showing that an excessive TGF-β signalling, due to reduced TGF-β sequestration within the defective OI ECM, is involved in the pathogenesis of OI (Grafe et al., 2014b). Abnormal collagen production and assembly have been shown to prevent proper retention of cytokines and proteoglycans within the bone matrix, causing a defective niche for the recruitment and differentiation of MSCs into osteoblasts (Kalajzic et al., 2002; Li et al., 2010b) It is therefore possible that AFSCs exert their beneficial effects in the *oim* model by contributing to the ECM structure. The production of a chimeric matrix of better quality compared to the defective *oim* ECM could be better able to sequester TGF-β and other factors, contributing to the maintenance of their physiological levels. Data collected for this thesis seem to support this hypothesis, since decreased gene expression levels of the TGF-β target *Serpine1* were observed in AFSC-transplanted mice compared to non-transplanted ones. Excessive TGF-β signalling has been shown to stimulate osteoclast activity and to prevent the differentiation of osteoblast, therefore reducing its levels might contribute to the improved *oim* pre-osteoblast differentiation observed following transplantation (Hattersley and Chambers, 1991; Erlebacher and Derynck, 1996; Alliston et al., 2001).

7.5 Limitations

The data described in this thesis represent a preliminary proof of concept for the use of amniotic fluid as a source of fetal MSCs for cell therapy in OI. This study however has some limitations that need to be addressed before the potential translation of AFSC-based therapy into clinical practice.

The most important limitation of this study is the small number of animals used for the *in vivo* experiments. The significance of some of the statistical analyses may have been limited by the modest number of sample used in this study. In particular, a *post hoc* power analysis on the micro-CT data showed that, based on the mean and effect size observed in the study (d=0.79), an n of 42 animals would be required to reach statistical power at 0.80 level.

Another important limitation is the lack of information on the long-term effects of AFSC transplantation. In this study, mice were culled and analysed at 8 weeks of age. Other preclinical studies using human fetal MSCs involved longer-term analyses, however never exceeding the 12 weeks, mainly due to regulations for the use of animals on research (Guillot et al., 2008a).

The only long-term data derived from the few clinical transplantations. Ten children affected by severe OI were transplanted with bone marrow MSCs and fetal liver MSCs (Horwitz et al., 2002; Le Blanc et al., 2005; Götherström et al., 2014). In all cases some therapeutic effects including boost in growth and reduction of fracture rate were seen, but was limited to the first months or year after surgery, leading to repeated transplantations. Moreover, in most cases the effects of MSC administration were confounded by the concomitant pharmacological treatment with bisphosphonates, making it difficult to discern the effects of the individual therapies. Longer-term studies with proper mutation-matched controls are needed not only to confirm the effectiveness of the therapy but also its safety, since the absence of *in vivo* teratomas formation was only investigated for a short time. It is however reassuring that no major adverse effects and no tumours were detected in children transplanted with

fetal MSCs, which suggests that the primitive phenotype of these cells does not pose a threat in terms of safety.

Another important issue that was not investigated in this study is the fate of those transplanted cells that remain trapped into the lungs, following systemic transplantation. It has been speculated that those cells, that are retained within the vasculature of the lungs due to their relative size, are able to influence distant tissues by secreting trophic factors, such as growth factors and cytokines, in the circulation. No preclinical studies of cell therapy for OI to date have investigated whether transplanted cells proliferate and differentiate in the lungs. It is however reassuring that fetal cell transplantation in children was well tolerated and no ectopic tissue formation was detected during follow up visits. This suggests that transplanted cells that are retained in the lungs do not proliferate excessively but rather undergo apoptosis or differentiate.

Finally, the exact mechanism of action of transplanted cells is also still largely unknown. While it is widely accepted that MSCs used in cell therapy may exert, at least in part, their therapeutic effects in a paracrine manner, through the secretion of numerous trophic factors and cytokines, little is known about the exact composition of the MSC "secretome" and especially of the portion which could be beneficial to the treatment of OI (Caplan and Dennis, 2006; Dimmeler et al., 2014).

Moreover, in this study, conditioned medium containing factors secreted by AFSCs was unable to reproduce the effects seen in the *in vivo* experiments, both when AFSCs were in their naïve state and when they were stimulated with TNFa and TGF-β. More studies on the paracrine effect of AFSCs are needed and particularly important would be the understanding of the exact signalling pathways impacted by the trophic factors released.

7.6 Future of cell therapy for OI

The current knowledge in terms of basic biology and technical progress is expanding quickly, opening new exciting scenarios in the field of cell therapy.

The further study and characterisation of the factors produced by fetal MSCs that contribute to ameliorating the OI disease phenotype could in the future allow the administration of purified factors as a pharmacological therapy. This strategy would allow researchers to mimic the beneficial effects seen after administration of stem cells, while avoiding any concerns related to the transplantation of whole cells and its long-term effects, including the possibility of immune response or ectopic tissue formation. Moreover, the administration of growth factors or cytokines is far less technically challenging than the processing and infusion of stem cells. This could facilitate the implementation of more frequent administrations, to keep the therapeutic effects constant and avoid the boosts of growth alternated with periods of growth decline seen in children transplanted with fetal MSCs (Horwitz et al., 2001; 2002; Le Blanc et al., 2005; Götherström et al., 2014). Moreover, MSCs used in cell-based therapies are considered by international regulatory bodies as "Advanced therapy medicinal products" and need to comply to strict Good Manufacturing Practice standards to ensure quality and safety of the product (Hanna et al., 2016).

More recently, the study of extracellular vesicles that are produced by MSCs and contain a variety of growth factors and cytokines has sparked interest for the potential application in regenerative medicine. The idea of a self-contained targeted delivery of trophic factors is appealing in the context of cell-free therapy for OI. It is thought that extracellular vesicles are unable to trigger any immune response and would facilitate the tight control of the dose and half-life of factors administered. The translation of extracellular vesicle therapies seems however to be far from implementation in clinical practice, mainly due to the difficulties on standardising the product and the high costs. Moreover, the European Medicinal Agency and the Food and Drug Administration have not yet classified the pharmaceutical category to which extracellular vesicles used for clinical applications might belong (Lener et al., 2015; Crivelli et al., 2017).

MSCs used so far in clinical cases of children with severe OI were transplanted in an allogeneic setting. They included adult bone marrow MSCs, which were obtained by HLA-matched siblings, and fetal liver MSCs, which were isolated by terminated pregnancies and were not HLA-matched (Horwitz et al., 1999; 2002; Le Blanc et al., 2005; Götherström et al., 2014). AFSCs can be isolated during pregnancy and at delivery, making it possible to use cells also in an autologous manner. It would be therefore possible to isolate AFSCs from an OI pregnancy and then genetically correct them and transplant them either in utero or postnatally ruling out any possibilities of immune recognition (Loukogeorgakis and De Coppi, 2016; 2017).

On the other hand, in line with the allogeneic use of MSCs that has been already investigated in the few cases to date of clinical transplantations in OI, biobanks of AFSCs would need to be built within GMP laboratories, to ensure adequate number of cells from the same donor in case of repeated transplantations needed. Moreover, with the advancement of scientific and technological knowledge, genome editing of cells could become a useful tool to gene-correct cells.

In terms of the best route of administration, it has recently become evident that *in utero* infusions, despite posing technical challenges, have many advantages over post-natal transplantation. Transplanting stem cells before birth could prevent *in utero* fractures and skeletal deformities that are often seen in the most severe cases of OI (Tonni et al., 2016). Moreover, studies have shown that developing tissues are more permissive to stem cell homing and support their survival and proliferation (Ramachandra et al., 2014). Lastly, smaller number of cells would be needed to transplant a fetus than a child, and this could shorten the time necessary to expand the number of cells needed, avoiding at the same time the pitfalls of culturing cells for long periods of time, which risks changing their phenotype and limits their proliferation and differentiation ability (Kim and Park, 2017).

The first clinical trial for cell therapy in OI, BOOSTB4, will in the upcoming years compare the outcomes of prenatal and postnatal fetal MSC transplantation in children with severe OI, including also for the first time a control group. The results

of the clinical trial will likely delineate the future of cell therapy strategies for OI, in terms of dose, timing and route of administration (Chitty et al., 2016).

7.7 Human fetal osteoblasts project

A side project of this thesis was the establishment of a protocol for the isolation and differentiation of human fetal osteoblasts. This project was initially started because of the difficulty in differentiating rodent osteoblasts, which require culturing cells in osteogenic permissive medium for up to four weeks. Moreover, there is a need to find a physiologically relevant cell type for the investigation of bone development and for the modelling of human bone, which could be useful for drug testing or to model bone diseases. Currently, most laboratories studying bone physiology and disease use rodent cells or human cell lines, such as the osteosarcoma cell lines MG-63 and Saos-2, which are however very different in their behaviour compared with human primary osteoblasts (Pautke et al., 2004). Human primary osteoblasts can be isolated by biopsies taken during orthopaedic surgery, however adult osteoblasts have very limited proliferation capacity and are not able to efficiently form bone *in vitro* (D'Ippolito et al., 1999; Gallagher, 2003). Fetal bones have been therefore investigated as a source of cells for the in vitro modelling of human bone (Gothard et al., 2015).

The protocol described in this thesis represents a promising source of human osteoblasts, that can be isolated from surgical waste from terminations of pregnancies. The protocol allows the isolation and expansion of a large number of cells, that do not lose their high proliferative capacity and differentiation potential even after repeated passaging and freezing-thawing cycles.

Thanks to the collaboration with the HDBR, a small biobank of fetal osteoblasts from different gestational ages, ranging from 9 to 21 weeks post conception has been developed and will keep expanding, with the aim of getting enough samples in triplicate for all gestational ages available. Possible applications that will be explored in the future include the further investigation of their *in vitro* bone differentiation

ability and how it changes across the different gestational ages. Moreover, a complete characterisation will be carried out, to fully assess the pattern of expression of osteogenic genes during fetal development.

In line with the interests of the laboratory for the study of OI, surgical waste from termination of OI pregnancies could be obtained and compared with age-matched samples from the cell bank. This will possibly contribute to the better understanding of the pathogenesis of OI, which is thought to start early in fetal skeletal development and could help identifying the best time for *in utero* transplantation of stem cells, in order to maximise the therapeutic outcomes.

Moreover, human fetal osteoblast cultures can be used to model human bone in the search for new drug treatments for skeletal disorders or in the study of potential bone side effects of other therapies, thus allowing the reduction of animals used and the related costs. The protocol presented in this thesis allows the robust differentiation of human fetal osteoblasts *in vitro* and offers an endpoint, bone formation, that is easily quantified.

7.8 Conclusions

In conclusion, work carried out for this thesis proposes AFSCs as a promising source of MSCs for cell therapy of OI. This work demonstrated that AFSCs transplanted into *oim* neonates led to therapeutic benefits and ameliorated the disease phenotype. Limited data from this study suggest that, beyond their potential as a source of replacement for defective OI osteoblasts, AFSCs may also mediate paracrine effects to stimulate endogenous osteoblast differentiation in the *oim* model. However, AFSC-conditioned medium was unable to replicate *in vitro* the effects seen *in vivo*. Larger studies are needed to further investigate the long-term safety and efficacy of these cells, as well as their mechanism of action, before the potential translation of AFSC-based therapies into clinical practice.

Abbreviations

αMEM	Alpha-modified essential medium
AFSC	Amniotic fluid stem cell
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BGLAP	Osteocalcin
BMD	Bone mineral density
BMP	Bone morphogenetic protein
Brtl IV	Brittle mouse IV
BSA	Bovine serum albumin
CaHA	Calcium hydroxyapatite
cDNA	Complementary DNA
CO_2	Carbon dioxide
COL1A1	Collagen type I alpha 1 chain
COL1A2	Collagen type I alpha 2 chain
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium

DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FLIM	Two photon lifetime imaging
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green fluorescent protein
GVHD	Graft versus host disease
HBSS	Hank's balanced salt solution
HDBR	Human Developmental Biology Resource
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
IFN γ	Interferon γ
iPSC	Induced pluripotent stem cell
IU	International unit
kDa	Kilo Dalton

M-CSF	Macrophage colony-forming factor
Micro-CT	Micro computed tomography
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
NIH	National Institutes of Health
OI	Osteogenesis imperfecta
Oim	Osteogenesis imperfecta murine
OPG	Osteoprotegerin
OPN	Osteopontin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR/RT-PCR	Quantitative/real time PCR
PFA	Paraformaldehyde
RANK	Receptor Activator of Nuclear Factor κ B
RANKL	RANK ligand
RNA	Ribonucleic acid
RNAse	Ribonucleasae
RUNX2	Runt-related transcription factor 2
SDF-1	Stromal cell-derived factor 1
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
TGF beta	Transforming growth factor β
TMD	Tissue mineral density
TNFα	Tumour necrosis factor α
Wpc	Weeks post conception
WT	Wild-type

Publications

First authored publications:

Ranzoni, A.M., Corcelli, M., Hau, K.L., Kerns, J.G., Vanleene, M., Shefelbine, S., Jones, G.N., Moschidou, D., Dala-Ali, B., Goodship, A.E., De Coppi, P., Arnett, T.R. and Guillot, P.V. "Counteracting bone fragility with human amniotic mesenchymal stem cells" *Scientific Reports* 6, 39656 (2016).

Ranzoni, A.M*., Corcelli, M*., Arnett, T.R. and Guillot, P.V. "Micro-computed tomography reconstructions of tibiae of stem cell transplanted osteogenesis imperfecta mice" (Accepted at *Scientific Data).*

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Hau, K.L., Ranzoni, A.M., Hawkins, K., De Coppi, P., David, A.L. and Guillot, P.V. "TGF-β-induced osteogenic potential of human amniotic fluid stem cells via CD73-generated adenosine production" *Scientific Reports* 7, 6601 (2017).

Hawkins, K.E., Corcelli, M., Dowding, K., Ranzoni, A.M., Vlahova, F., Hau, K.L., Hunjan, A., Hristova, M. and Guillot, P.V. "Embryonic stem cell-derived MSCs have a superior neuroprotective capacity over fetal MSCs in the hypoxic-ischemic mouse brain" *Stem Cells and Translational Medicine* 23, 541 (2018)

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